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Izuho Hatada *Editor*

Genome Editing in Animals

Methods and Protocols

Second Edition

Humana Press

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Edited by

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Preface

Conventional gene targeting is widely used to generate knockout mice. However, the conventional method depends on a rare event, spontaneous homologous recombination. This makes it impossible to produce gene-targeted mice directly from fertilized eggs, which in any case are limited in numbers for use in experiments. Consequently, for conventional gene targeting, embryonic stem cells (ESCs) are used instead of fertilized eggs because the former can be easily propagated. However, because generation of germline-competent ESCs is extremely difficult, conventional gene targeting is only possible for mice and rats, for which ESCs are available. This problem was solved by the advent of genome editing technologies, which enable specific cleavage of targeted loci, frequently followed by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ results in a small deletion or insertion at the target locus, whereas HDR can yield a knock-in at the target locus if a donor DNA is provided. There are three genome editing technologies: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system.

Genome Editing in Animals: Methods and Protocols aims to collect protocols that can be used for generation of knockout animals. Five years have passed since the first edition was published. During this period, there have been many technological advances. Therefore, in the second edition, the number of chapters has been significantly increased to 29, compared to 20 in the first edition. The first four chapters introduce basic protocols for genome editing technologies that can be applied to all animals. Chapter 5 introduces target design tools that can also be used in all animals. Starting in Chapter 6, specific protocols for each animal are provided: mouse (Chapters 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16), rat (Chapters 17 and 18), hamster (Chapter 19), rabbit (Chapter 20), pig (Chapters 21 and 22), chicken (Chapter 23), zebra fish (Chapter 24), medaka (Chapter 25), *Xenopus* (Chapter 26), silkworm (Chapter 27), sea squirt (Chapter 28), and *C. elegans* (Chapter 29).

I thank all of the authors for their outstanding contributions to this volume. On behalf of all of us, I hope that our effort to make these methods accessible will prove useful to genome editing aficionados around the world.

Maebashi, Gunma, Japan ***Izuho Hatada***

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Chapter 1

Construction and Evaluation of Zinc Finger Nucleases

Hiroshi Ochiai and Takashi Yamamoto

Abstract

Zinc finger nucleases (ZFNs) are programmable nucleases that have contributed significantly to past genome-editing research. They are now utilized much less owing to the advent of transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats and CRISPR-associated protein system (CRISPR-Cas). These new methods allow for easier generation of reagents that target genomic sequences of interest and are less labor-intensive than ZFNs at targeting desired sequences. However, fundamental ZFN patents have expired, enabling a wide range of their distribution for clinical and industrial applications. This article introduces a ZFN construction protocol that uses bacterial one-hybrid (B1H) selection and single-strand annealing (SSA) assay.

Key words Zinc finger nuclease, Genome editing, Bacterial one-hybrid system, Single-stranded annealing assay, PCR-based assembly

1 Introduction

Zinc finger nucleases (ZFNs) are programmable nucleases that have opened the door to the genome-editing era [1]. ZFN is an artificial protein consisting of a C_2H_2 zinc finger array (ZFA) DNA-binding domain and the nuclease domain of the FokI restriction enzyme. When a pair of ZFNs binds to DNA in a tail-to-tail configuration, the two FokI nuclease domains dimerize and induce a DNA double-strand break (DSB) (Fig. 1a). Since a single zinc finger domain can recognize a 3-nucleotide sequence, a pair of 3-finger (three-domain) nucleases can recognize an 18-nucleotide sequence. This recognition ability means that the pair could recognize one sequence out of 6.8×10^{10} bp, implying the ability to target a unique sequence in a eukaryotic genome. As reviewed elsewhere, DSBs introduced by programmable endonucleases are repaired through nonhomologous end joining (NHEJ) or homology-directed repair (HDR). Exploiting these endogenous repair pathways, researchers can edit genomic sequences [2].

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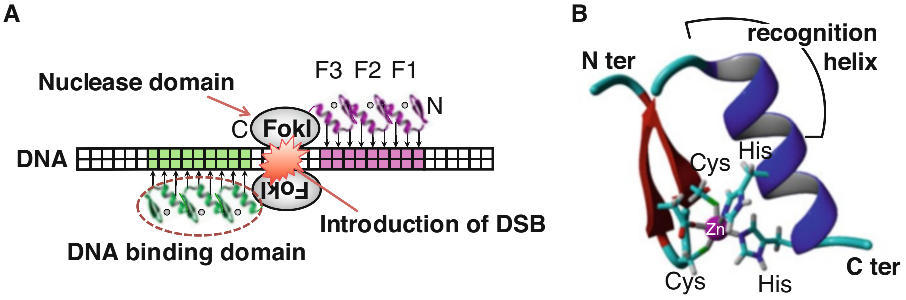


Fig. 1 Introduction of DNA double-strand break by ZFNs. (**a**) Schematic representation of ZFNs. Each ZF domain is referred to as F1, F2, or F3, depending on its position. (**b**) Schematic representation of a C_2H_2 ZF domain

PGEKPYKCPECGKSFSTSGHLVRHQRTH

PGEKPYKCPECGKSFSXXXXLXXHQRTH

Fig. 2 Partial randomization of recognition helix. Primary sequence of a ZF domain. Blue and pink letters represent helix residues participating in zinc coordination and nucleotide recognition, respectively. Residues in the recognition helix are subjected to partial randomization. X is one of all possible amino acid residues except for C, F, Y, or W; this randomization is achieved by using codon VNS

The construction method for sequence-specific ZFNs is laborious and has not been widely used after the advent of transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats-CRISPR-associated protein system (CRISPR-Cas). ZFN proteins are more compact in size than TALENs and CRISPR-Cas systems. Furthermore, fundamental ZFN patents have expired, and this fact may encourage a wide range of their implementation for clinical and industrial applications [3]. This article introduces a ZFN construction protocol that uses bacterial one-hybrid (B1H) selection and single strand annealing (SSA) assay [4].

The C_2H_2 zinc finger domain is a peptide comprised of ~30 amino acids (aa). It contains two β-strands and one α-helix (Fig. 1b) [5, 6]. It has been well known that amino acids beside α-helix (−1 to +6 aa from the start aa of α-helix) recognize a three-nucleotide sequence (subsite) (Fig. 1). Therefore, the α-helix is referred to as the recognition helix. To engineer a ZFA to recognize a sequence of interest, ZF libraries with partially randomized recognition helices are constructed (Fig. 2). Next, by using a bacterial one-hybrid system, a ZFA binding specifically to the target sequence is selected (*see* below).

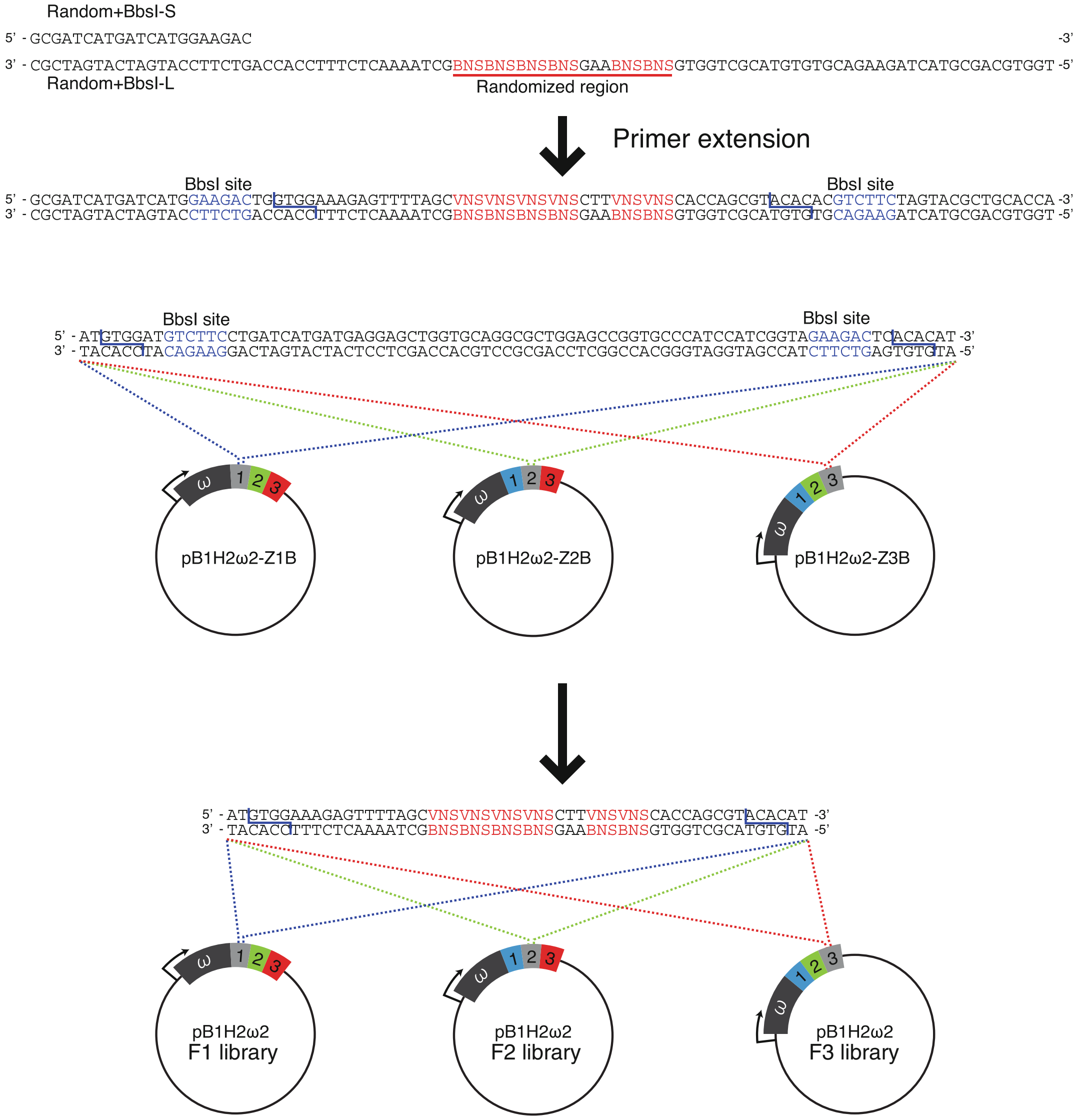

Random+BbsI-S
5' - GCGATCATGATCATGGAAGAC -3'
3' - CGCTAGTACTAGTACCTTCTGACCACCTTTCTCAAAATCGBNSBNSBNSBNSGAABNSBNSGTGGTCGCATGTGTGCAGAAGATCATGCGACGTGGT -5'
Random+BbsI-L
Randomized region
Primer extension
BbsI site
BbsI site
5' - GCGATCATGATCATGGAAGACTGGTGGAAAGAGTTTTAGCVNSVNSVNSVNSCTTVNSVNSCACCAGCGTACACACGTCTTCTAGTACGCTGCACCA -3'
3' - CGCTAGTACTAGTACCTTCTGACCACCTTTCTCAAAATCGBNSBNSBNSBNSGAABNSBNSGTGGTCGCATGTGTGCAGAAGATCATGCGACGTGGT -5'
BbsI site
BbsI site
5' - ATGTGGATGTCTTCCTGATCATGATGAGGAGCTGGTGCAGGCGCTGGAGCCGGTGCCCATCCATCGGTAGAAGACTCACACAT -3'
3' - TACACCTACAGAAGGACTAGTACTACTCCTCGACCACGTCCGCGACCTCGGCCACGGGTAGGTAGCCATCTTCTGAGTGTGTA -5'
ω
1
2
3
pB1H2ω2-Z1B
pB1H2ω2-Z2B
pB1H2ω2-Z3B
5' - ATGTGGAAAGAGTTTTAGCVNSVNSVNSVNSCTTVNSVNSCACCAGCGTACACAT -3'
3' - TACACCTTTCTCAAAATCGBNSBNSBNSBNSGAABNSBNSGTGGTCGCATGTGTA -5'
pB1H2ω2
F1 library
pB1H2ω2
F2 library
pB1H2ω2
F3 library

Fig. 3 Construction of randomized ZF library. Schematic representation of randomized ZF library construction

A randomized library is constructed using a long degenerate base-containing oligo (Random+BbsI-L) and a primer (Random+BbsI-S) (Fig. 3). The degenerate base-containing oligo is converted to double-strand DNA by primer extension. Next, using appropriate restriction enzymes, three randomized libraries are constructed (Fig. 3). Each library corresponds to one randomized ZF for the F1, F2, or F3 position. Next, the randomized libraries are transformed into US0*ΔhisBΔpyrFΔrpoZ* bacteria containing pH3U3 reporter plasmid (Fig. 4). The US0*ΔhisBΔpyrFΔrpoZ* bacterial strain does not have a functional *HisB*, which encodes an enzyme essential for histidine synthesis; the strain cannot survive

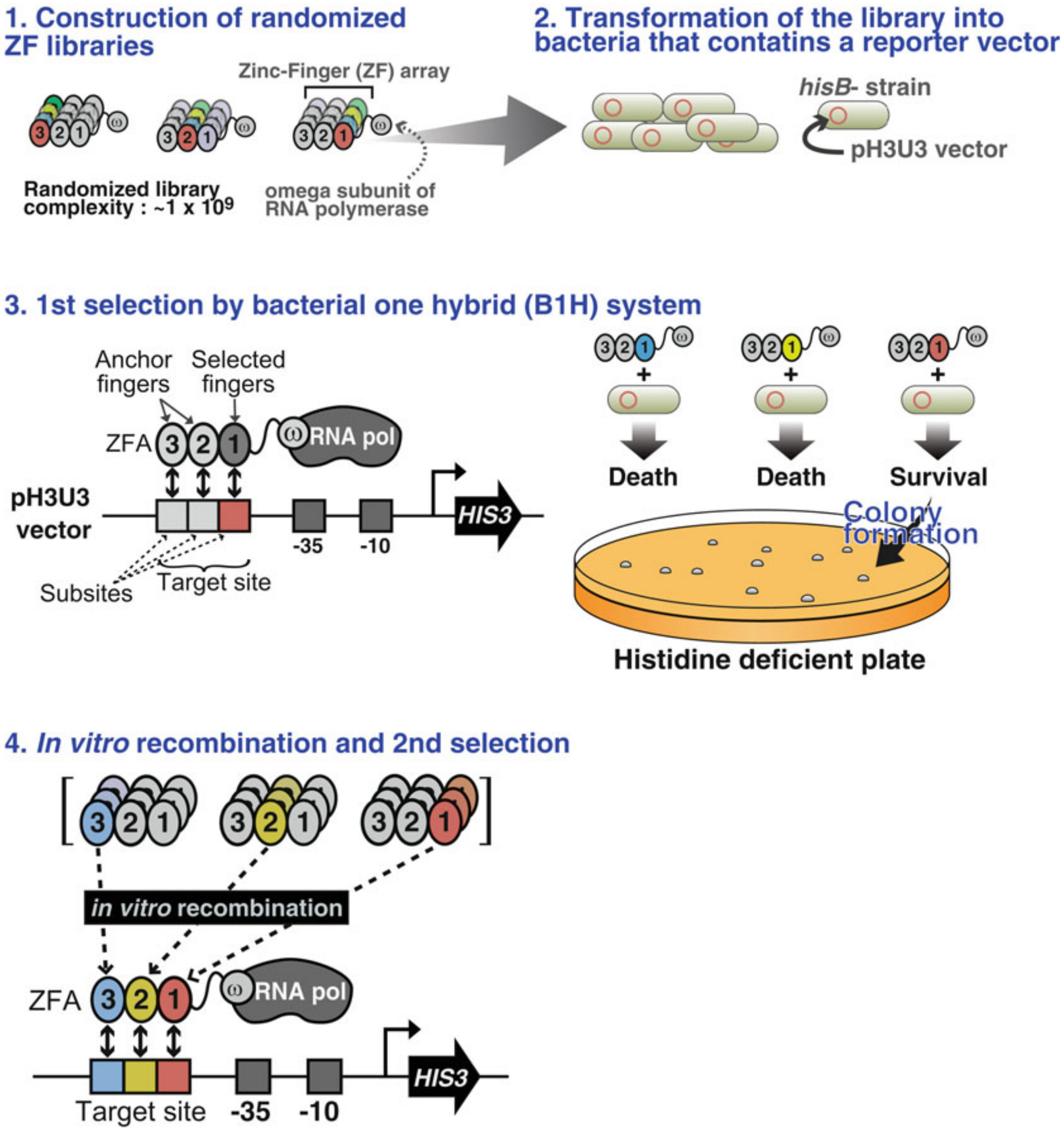

1. Construction of randomized ZF libraries
Zinc-Finger (ZF) array
3 2 1 ω
Randomized library complexity : ~1 x 10^9
omega subunit of RNA polymerase
2. Transformation of the library into bacteria that contatins a reporter vector
hisB- strain
pH3U3 vector
3. 1st selection by bacterial one hybrid (B1H) system
Anchor fingers
Selected fingers
ZFA
ω RNA pol
pH3U3 vector
-35
-10
HIS3
Subsites
Target site
Death
Death
Survival
Colony formation
Histidine deficient plate
4. In vitro recombination and 2nd selection
in vitro recombination
ZFA
ω RNA pol
HIS3
Target site
-35
-10

Fig. 4 Directed domain shuffling and bacterial one-hybrid system. Schematic representation of functional ZFA selection using a bacterial one-hybrid system

on a histidine-free medium. Meanwhile, the pH3U3 reporter plasmid contains yeast-derived *His3* with a basal promoter and an upstream zinc finger target site. The bacteria express the randomized library, producing fusion proteins of RNA-polymerase ω subunit and ZFA with a randomized finger. If a ZFA-RNA polymerase ω subunit binds to the target site, the bacteria express HIS3 and survive on histidine-free medium (Fig. 4). By using the HIS3-specific inhibitor 3-amino-1,2,4-triazole (3-AT), the selection intensity can be adjusted. In the first selection, three sub-selected

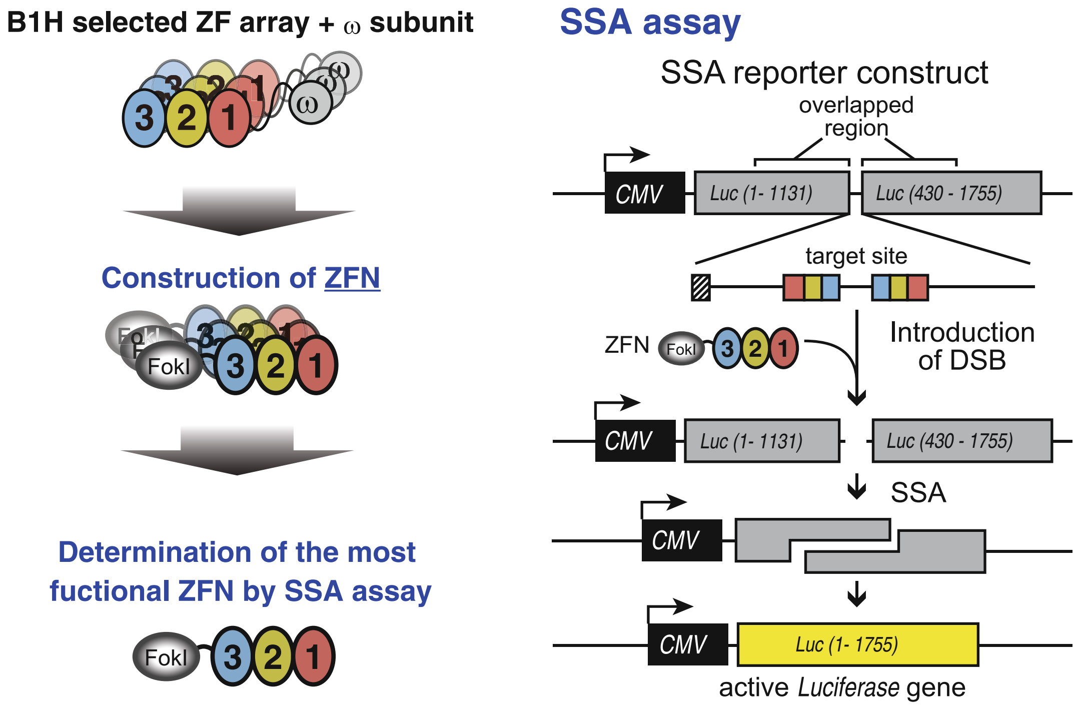


Fig. 5 SSA assay. Schematic representation of SSA assay

ZF libraries are obtained. Using PCR-based assembly, sub-selected ZF libraries are combined. Next, using this new library, a second selection with a higher selection pressure is performed, to obtain a three-finger ZF recognizing the target sequence.

To test the function of ZFNs containing ZFA DNA-binding domains selected by B1H, single-strand annealing (SSA) assays are performed (Fig. 5). At first, ZFN expression and SSA reporter vectors are transfected into HEK293T cells. The reporter plasmid contains ZFN target sites between two split and inactive parts of the luciferase gene, with overlapping repeated sequences. Following a DSB caused by a functional ZFN, a functional luciferase gene is generated by an SSA reaction (Fig. 5). Zinc finger nuclease functionality can therefore be quantified by the luciferase activity.

2 Materials

2.1 Plasmids and Bacterial Strains

1. pB1H2ω2-Z1B [4]: low copy plasmid, ampicillin resistant.
2. pB1H2ω2-Z2B [4]: low copy plasmid, ampicillin resistant.
3. pB1H2ω2-Z3B [4]: low copy plasmid, ampicillin resistant.
4. pH3U3-mcs (Addgene plasmids 12609): low copy plasmid, kanamycin resistant.

5. pB1H2ω2+BbsI [4]: low copy plasmid, ampicillin resistant.
6. pSTL plasmid [4]: ampicillin resistant.
7. pSTL-ZFA36 [7]: ampicillin resistant.
8. pGL4-SSA [4]: ampicillin resistant.
9. pGL4-SSA-ZFA36 [7]: ampicillin resistant.
10. pRL-CMV (Promega): ampicillin resistant.
11. XL1-Blue Electroporation-Competent Cells (Agilent, 200228).
12. US0Δ*his*BΔ*pyrF*Δ*rpoZ*: [F′ *lacIqZ*Δ*MI5 Tn10(Tetr)*] (Addgene plasmids 18049): tetracycline resistant.
13. HEK293T cell.

2.2 Primers

1. Random+BbsI-S: 5′-GCGATCATGATCATGGAAGAC-3′.
2. Random+BbsI-L: 5′-TGGTGCAGCGTACTAGAAGACGTGTGTACGCTGGTGSNBSNBAAGSNBSNBSNBSNBGCTAAAACTCTTTCCACCAGTCTTCCATGATCATGATCGC-3′.
3. HIS3 rev seq: 5′-GATCGAGTGCTCTATCGCTA-3′.
4. ZF1-forward: 5′-AAGGTCGTGCGGCCGGAAGACAAGCCTTACAAATGCCCAGAA-3′.
5. ZF1-reverse: 5′- CAGGACACTTATAGGGTTTTCCCCGGTATGTGTA-3′.
6. ZF2-forward: 5′-GAAAAACCCTATAAGTGTCCT-3′.
7. ZF2-reverse: 5′-CCGGGCACTTGTATGGCTTCTCCCCGGTATGTGTA-3′.
8. ZF3-forward: 5′-GAGAAGCCATACAAGTGCCCG-3′.
9. ZF3-reverse: 5′- GACTTGTCGGCCTTGAAGACGTCTCTAGTGTGCAGAGGATCCACGCAGG-3′.
10. pB1H2ω2-seq-F: 5′-GATCCTCGACGTTCGCGAAC-3′.
11. pB1H2ω2-seq-R: 5′-GCTGCGCAACTGTTGGGAAG-3′.
12. T7 promoter primer: 5′-TAATACGACTCACTATAGGG-3′.
13. S418-R: 5′-ATTTCTGGCAATTTCAATTAATTC-3′.

2.3 Enzymes

1. KOD -Plus- (TOYOBO).
2. Restriction enzymes: BbsI, EcoRI, XmaI, XbaI, BsgI, BsaI, KpnI, and SacI.
3. T4 DNA ligase.
4. rAPid alkaline phosphatase (Roche).

2.4 Instruments and Kits

1. Dialysis membrane.
2. Gene Pulser Electroporator (BIORAD).
3. 1 mm electroporation cuvette.

4. 150 × 15 mm round culture dish.
5. 1 mm glass beads.
6. Gel Indicator DNA Extraction Kit (Biodynamics).
7. Ultrafiltration filter, Amicon Ultra-0.5, 10k (Millipore).
8. QIAGEN Plasmid Midi Kit (QIAGEN).
9. QIAGEN Gel Extraction Kit (QIAGEN).
10. QIAGEN PCR Purification Kit (QIAGEN).
11. NuSieve 3:1 agarose (Lonza).
12. NuSieve GTG agarose (Lonza).
13. Agarose.
14. Ligation-Convenience Kit (NIPPON GENE).
15. Dual-Glo luciferase assay system (Promega).
16. 96-well flat bottom cell culture plate.
17. 96-well solid white flat bottom polystyrene plate.
18. Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific).

2.5 Medium and Buffers

1. 0.5 × TBE.
2. 3 M sodium acetate (pH 5.2).
3. Ethanol.
4. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM of Tris–HCl, pH 8.0, 1 mM of EDTA.
5. Chloroform.
6. Autoclaved double-distilled water (ddH_2O).
7. 10% glycerol.
8. 25 mg/mL ampicillin.
9. 30 mg/mL kanamycin.
10. 5 mg/mL tetracycline in EtOH.
11. 50 mg/mL carbenicillin.
12. Transformation buffer for chemically competent cell preparation: Dissolve 0.6 g of PIPES and 3.72 g of KCl into 170 mL of ddH_2O. Adjust the solution to pH 6.7 using 5 N of KOH. Add ddH_2O up to 190 mL, and autoclave the solution. Dissolve 0.528 g of $CaCl_2 \cdot 2H_2O$ and 2.616 g of $MnCl_2 \cdot 4H_2O$ into 12 mL of ddH_2O. Sterilize the solution using a 0.22 μm filter. Add 10 mL of $CaCl_2$/$MnCl_2$ solution into autoclaved solution. Store at 4 °C.
13. DMSO.
14. 10× annealing buffer [8]: 400 mM Tris–HCl (pH 8), 200 mM $MgCl_2$, 500 mM NaCl.

15. 2× YT: 16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl; adjust pH to 7.0 ± 0.2 (25 °C) with 10 N NaOH (for 500 mL 2× YT medium preparation, ~200 μL of 10-N NaOH is required). Autoclave to sterilize.
16. LB: 10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl; adjust pH to 7.5 with 10 N NaOH. Sterilize by autoclaving.
17. SOB: 20 g of Bacto-tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 mL of 1 M KCl, ddH_2O to 1000 mL; adjust pH to 7.0 with 10 N NaOH; autoclave to sterilize. Add 2 mL of 1 M $MgCl_2$.
18. SOC: 20 g of Bacto-tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 mL of 1 M KCl, ddH_2O to 1000 mL; adjust pH to 7.0 with 10 N NaOH; autoclave to sterilize. Add 20 mL of sterile 1 M glucose and 2 mL of 1 M $MgCl_2$.
19. NM medium [9]: 1× M9 salts (*see* below), 1× amino acid mixture containing 17 of the 20 amino acids (*see* below), 40 mg/mL glucose, 10 μg/mL thiamine, 200 μM uracil (*see* **Note 1**), 200 μM adenine (*see* **Note 1**), 20 μM $ZnSO_4$, 100 μM $CaCl_2$, and 1 mM $MgSO_4$. Filter sterilize through a 0.22 μm filter (Corning bottle-top vacuum filter system). NM medium can be stored at 4 °C for ~6 months.
20. 20× M9 salts: Dissolve 13.6 g $Na_2HPO_4{\cdot}7H_2O$, 6 g KH_2PO_4, 1 g NaCl, and 2 g NH_4Cl, sequentially, in 90 mL of distilled water. Make up the volume to 100 mL with distilled water. Sterilize by autoclaving, and store at room temperature.
21. 33.3× amino acid mixture [9]: Amino acid mixture containing 17 of the 20 amino acids (omitting His, Met, and Cys). Prepare the following six solutions (all percentages are wt/vol). Mix equal volumes of all six solutions to get 33.3× amino acid mixture. Filter sterilize through a 0.22 μm filter (Corning bottle-top vacuum filter system), and store at 4 °C for up to 6 months.
    - Solution I (200×): dissolve 0.99 g Phe (0.99%), 1.1 g Lys (1.1%), and 2.5 g Arg (2.5%) in water (100 mL final volume).
    - Solution II (200×): Dissolve 0.2 g of Gly (0.2%), 0.7 g of Val (0.7%), 0.84 g of Ala (0.84%), and 0.41 g of Trp (0.41%) in water (100 mL final volume).
    - Solution III (200×): Dissolve 0.71 g of Thr (0.71%), 8.4 g of Ser (8.4%), 4.6 g of Pro (4.6%), and 0.96 g of Asn (0.96%) in water (100 mL final volume).
    - Solution IV (200×): Add 9.1 mL of 36.5% HCl to 80 mL of water, and subsequently dissolve 1.04 g of Asp (free acid, 1.04%) and 14.6 g Gln (14.6%) into the solution (100 mL final volume).

- Solution V (200×): Dissolve 18.7 g of potassium-Glu (18.7%) in 80 mL of water, subsequently add 0.36 g of Tyr (0.36%) and 4 g of NaOH pellets, and stir to dissolve (100 mL final volume).
- Solution VI (200×): Dissolve 0.79 g of Ile (0.79%) and 0.77 g of Leu (0.77%) in water (100 mL final volume).

22. 3-AT selection plate (500 mL) [9]: Autoclave 9 g Bacto agar in 438 mL of ddH_2O with a stir bar. Stir agar while cooling to ~55 °C. Combine the solutions in the recipe below in order, and add to the agar; 25 mL of 20× M9-salt solution, 10 mL of 20% (wt/vol) glucose, 5 mL of 20 mM adenine, 15 mL of 33.3× amino acid mixture, 5 mL of 20 mM uracil, 0.5 mL of 0.1 M $CaCl_2$, 0.5 mL of 1 M $MgSO_4$ and 0.5 mL of 10 mM $ZnSO_4$. Next, add 50 μL of 100 mM IPTG (10 μM final concentration), 5–10 mL of 1 M 3-AT (depending on the final desired 3-AT concentration), 0.5 mL of 25 mg/mL kanamycin (50 μg/mL final concentration), and 1 mL 50 mg/mL carbenicillin (100 μg/mL final concentration) to the agar while stirring (500 mL final volume). Pour 50 mL into each 150 × 15 mm round culture dish. AT selection plates are good for only ~3 weeks when stored at 4 °C.
23. Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific).
24. Growth medium: D-MEM with 10% fetal bovine serum (FBS).

3 Methods

3.1 Construction of the Randomized ZFN Library

3.1.1 Primer Extension

1. For primer extension, assemble the following reaction in a PCR tube: 15 μL of 2 mM dNTPs, 15 μL of 10 × Buffer for KOD -Plus-, 60 μL of 50 μM Random+BbsI-S, 20 μL of 50 μM Random+BbsI-L, 6 μL of 25 mM $MgSO_4$, 3 μL of KOD -Plus-, and 31 μL of ddH_2O. Reaction conditions are 95 °C, 5 min; 50 °C, 5 min; and 68 °C, 30 min.
2. Add an appropriate amount of loading dye to the reaction solution. Load all of the solution into ten separate wells of a 3% NuSieve GTG agarose gel, and run the gel at 100 V for 35 min. Stain DNA with suitable DNA-staining solution; collect the band of interest. Purify DNA fragments using electroelution, as described below (*see* **Note 2**).

3.1.2 Fragment Purification by Electroelution

1. Place the excised gel in a dialysis membrane tube. Next, add 800 μL 0.5× TBE (or 1× TAE).
2. Close the dialysis membrane tube with clips. Remove the air as much as possible.

3. Put the tube in an electrophoresis tank containing 0.5× TBE or 1× TAE buffer. Electrophorese at 100 V for 40 min to elute DNA fragments.
4. Carefully remove the clip on one side, recover the DNA solution using a pipette, and transfer it to a 1.5 mL tube.
5. Centrifuge the tube at 18,000 × *g*, 22 °C for 5 min to pellet residual agarose gel particles.
6. Aliquot 400 μL of supernatant into each of two 1.5 mL tubes, add 1/10 volume of 3 M sodium acetate, and mix well. Add 800 μL of 100% ethanol to each tube, and mix well. Incubate the tubes on ice for 10 min.
7. Centrifuge at 16,000 × *g*, 4 °C, for 15 min.
8. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.
9. Dry the pellet in vacuum for 5 min. Dissolve it in 20 μL of ddH_2O, and quantify the DNA concentration. Store at −20 °C.

3.1.3 BbsI Treatment

1. Treat the purified fragment with BbsI by using the following reaction composition: 50 μL of DNA solution, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O up to 100 μL.
2. Incubate at 37 °C for 3 h or more (overnight, if possible).
3. Add an appropriate amount of loading buffer to the reaction solution. Next, electrophorese the entire solution in ten separate lanes of a 3% NuSieve GTG agarose gel. Electrophorese Random+BbsI-L oligo in one lane of the same gel. After electrophoresis and appropriate DNA dye staining, excise the band of interest (47 bp randomized fragment). DNA fragments are purified by electroelution, as above (*see* **Note 2**).
4. Treat plasmid vectors for the insertion of the randomized fragment (pB1H2ω2-Z1B, pB1H2ω2-Z2B, and pB1H2ω2-Z3B) [4] by BbsI as follows. Mix 30–50 μg of plasmid vector, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O up to 100 μL. Incubate the mixture at 37 °C for 3 h or more (overnight, if possible).
5. Electrophorese reaction on a 1% agarose gel, and purify backbone fragments using a QIAGEN Gel Extraction Kit. Next, perform ethanol precipitation, and dissolve the precipitate in ddH_2O, to 100 ng/μL. In addition, excise the smaller fragments, and recover them by electroelution (Subheading 3.1.2). The smaller fragments can be used as a positive control at the ligation step.

Table 1
Ligation reaction

	Negative control (μL)	Sample 1 (μL)	Sample 2 (μL)	Sample 3 (μL)
pB1H2ω2-Z(1–3)B (100 ng/μL)	1	1	1	1
Randomized fragment (10 ng/μL)	0	0.5	1	2.5
10× ligation buffer	1	1	1	1
T4 DNA ligase	0.2	0.2	0.2	0.2
ddH_2O	7.8	7.3	6.8	5.3

3.1.4 Small-Scale Ligation

1. Find the optimal concentration ratio of vector and insert in ligation reactions. Prepare four reaction solutions as described by Table 1.
2. Incubate at 16 °C for 4 h or more.
3. After the incubation, the reaction solution of each 1 μL is electroporated into 60 μL of XL1-Blue electro-competent cells in a 1 mm cuvette (1.75 kV, 25 μF, 200 Ω in a Gene Pulser Electroporator). Immediately transfer the electroporated cells to 940 μL of 37 °C pre-warmed SOC, and culture with shaking at 37 °C for 1 h. Prepare four 1.5 mL tubes, each containing 45 μL of LB medium. After incubation, take 5 μL of cultured medium, transfer to an LB containing tube, and mix well. Next, transfer 5 μL of the diluted culture to another LB-containing tube; mix well. Repeat this procedure for the remaining tubes. Plate 5 μL each of diluted culture onto 10 cm LB agar plates containing ampicillin. Incubate overnight at 37 °C.
4. On the following day, count the number of colonies. Depending on the dilution ratio (2×10^{-3}, 2×10^{-4}, 2×10^{-5}, 2×10^{-6}, or 2×10^{-7}), determine the transformation efficiency. The transformation efficiency of a ligation product containing a randomized fragment should be at least tenfold that of empty vector (negative control). The ideal transformation efficiency of ligation product with insert is 10^4–10^5. If the number of colonies for ligation product with a randomized fragment is about the same as it is for the negative control, then it is possible that the randomized fragment preparation is not going well. In this case, perform a ligation reaction with the positive control fragment prepared in Subheading 3.1.3. If this positive control exhibits an increase in the number of colonies,

but the randomized fragment insert does not, the randomized fragment preparation failed. In this case, prepare new randomized fragments.

3.1.5 Large-Scale Ligation

1. At the optimum concentration ratio of vector and insert, perform the ligation on a large scale, as follows: 100 μL of pB1H2ω2-Z(1–3)B (100 ng/μL), appropriate amount of randomized fragment (50 ng/μL), 20 μL of 10× ligation buffer, 10 μL of T4 DNA ligase, and ddH_2O up to 200 μL.
2. Incubate at 16 °C for overnight.
3. Add 20 μL of 3 M sodium acetate, and mix well. Next, add 220 μL of phenol:chloroform:isoamyl alcohol; vortex for 30 s. Centrifuge at 14,000 × *g* at room temperature for 5 min.
4. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 220 μL of chloroform; vortex for 30 s. Centrifuge for 5 min at 14,000 × *g* and room temperature.
5. Repeat **step 4**.
6. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 500 μL of 100% ethanol; mix well. Incubate at −20 °C for at least 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C.
7. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.
8. Dry the pellet in vacuum for 5 min. Dissolve it in 24 μL of ddH_2O. Store at −20 °C.

3.1.6 Electroporation

1. Add 24 μL of the purified ligation product into 1.2 mL of XL1-Blue electrocompetent cells. Gently mix the tube, and put it on ice. Transfer 60 μL of this mixture into an ice-cold electroporation cuvette, and perform electroporation as described above (Subheading 3.1.4). Immediately transfer the electroporated cells into pre-warmed 1 mL SOC. Repeat this procedure 20 times.
2. Transfer all electroporated cells to the 500 mL flask containing 100 mL of pre-warmed SOC. Agitate for 1 h at 80 rpm and 37 °C.
3. Using 20 μL of culture, predict the number of total transformants (*see* Subheading 3.1.4).
4. Add 150 mL of 2× YT medium and ampicillin to a final concentration to 100 μg/mL; agitate for 3 h at 80 rpm and 37 °C. As described above, using 20 μL of culture, predict the number of expanded transformants (*see* Subheading 3.1.4). The number of transformants should proliferate by 5–10-fold compared to that before 3 h incubation.

5. Dispense bacterial cultures into two pre-chilled 50 mL tubes. Centrifuge at 4000 × *g*, at 4 °C for 15 min. As possible, discard supernatant.
6. Add 25 mL each of remaining bacterial cultures into individual tubes. Centrifuge at 1200 × *g*, at 4 °C for 10 min. As possible, discard supernatant.
7. Add 10 mL of ice-cold 15% glycerol to one of tubes. Gently pipet to completely suspend the cells. Flash-freeze the tube in liquid nitrogen, and store it at −80 °C.
8. Purify plasmids from the other tube using a QIAGEN Plasmid Midi Kit. Adjust the plasmid concentration to 100 ng/μL in ddH_2O. The obtained plasmid solution can be stored at −20 °C. (Keep in mind that pB1H2ω2 is a low copy number plasmid. The typical amount of purified plasmid is ~8 μg.)

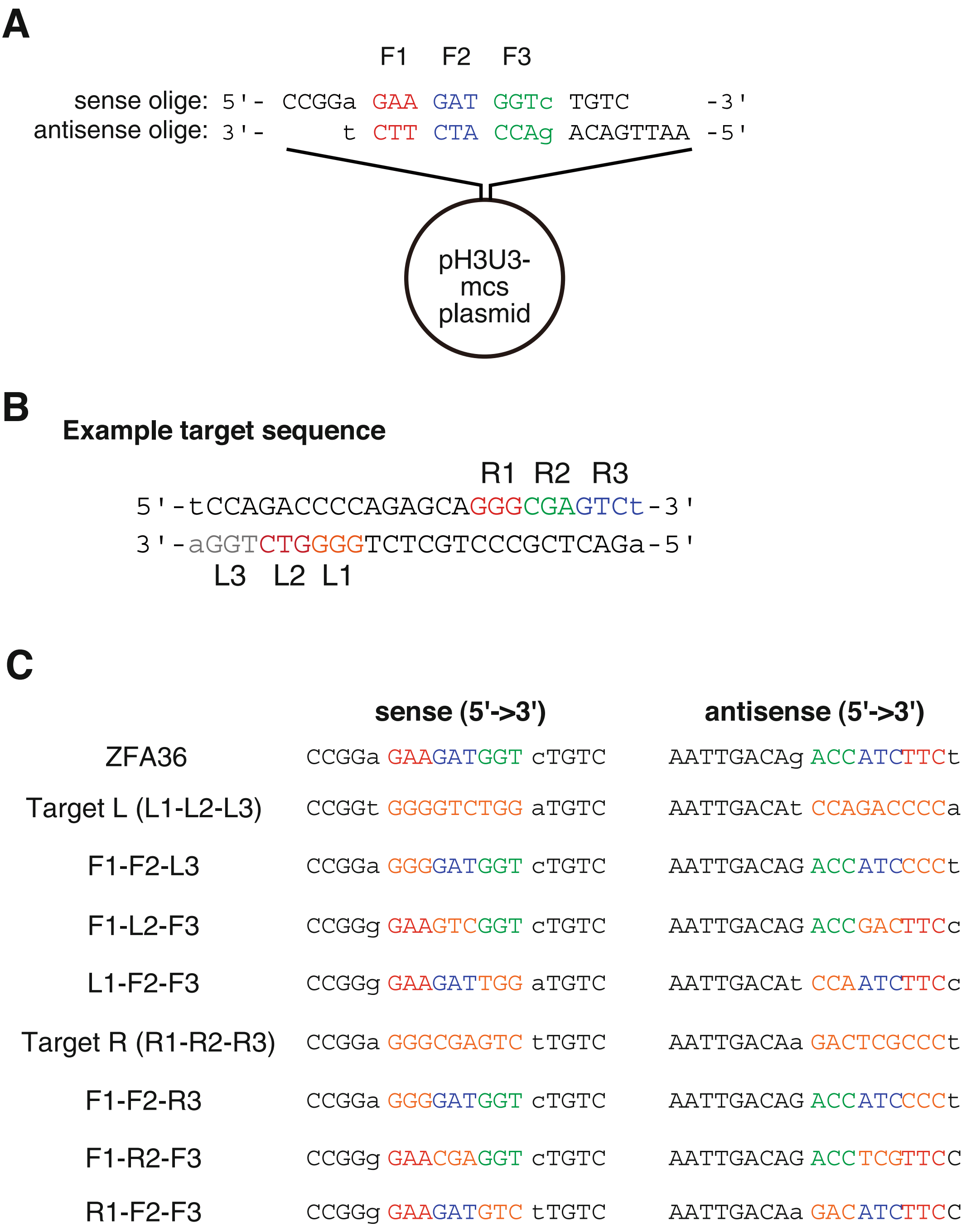
3.2 First B1H Selection

3.2.1 Target Sequence Determination

1. Determine a target sequence. The target sequence should contain a 6 bp spacer sequence. Each ZF binding subsite should have the motif RNN, that is, the target sequence should satisfy the pattern 5′-NNYNNYNNYNNNNNNRNNRNNRNN-3′ [10].

3.2.2 Construction of pH3U3-Target Vector

1. In the first randomized libraries, non-randomized ZF domains are derived from ZFA36, whose binding sequence is GAAGATGGTc [4, 11, 12]. Therefore, in an F3 library, the target sequence should satisfy the pattern XXXGATGGTc, where XXX is the target subsite of the final target sequence.
2. A three-finger ZFN contains a ZFA and a nuclease domain at its N and C termini, respectively. Fingers from N terminus are referred to as F1, F2, and F3, respectively. The F3 and F1 ZF domains recognize subsites at the 5′ and 3′ sides, respectively (Figs. 1 and 6).
3. Individual ZF domains tend to recognize not only their corresponding subsite but also a nucleotide adjacent to the 3′ side of the subsite [5]. Therefore, the target subsite for F1 ZF should be XXXx (XXX and x are the final target subsite and the additional 3′ nucleotide, respectively).
4. As described in Fig. 6, prepare oligos containing ZF target sites.
5. Sequentially digest 200 ng of pH3U3-mcs by EcoRI and XmaI. After electrophoresis, excise the band of interest (~5.8 kbp), and purify the DNA using a QIAGEN gel extraction kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
6. Prepare designed oligos from the appropriate manufacturer. Prepare reaction mixture as follows: 1 μL of 10× annealing buffer, 1 μL of B1H sense oligo (50 μM), 1 μL of B1H antisense oligo (50 μM), and 7 μL of ddH_2O.


A
F1 F2 F3
sense olige: 5'- CCGGa GAA GAT GGTc TGTC -3'
antisense olige: 3'- t CTT CTA CCAg ACAGTTAA -5'
pH3U3-mcs plasmid
B
Example target sequence
R1 R2 R3
5'-tCCAGACCCCAGAGCAGGGCGAGTCt-3'
3'-aGGTCTGGGGTCTCGTCCCGCTCAGa-5'
L3 L2 L1
C

	sense (5'->3')	antisense (5'->3')
ZFA36	CCGGa GAAGATGGT cTGTC	AATTGACAg ACCATCTTCt
Target L (L1-L2-L3)	CCGGt GGGGTCTGG aTGTC	AATTGACAt CCAGACCCCa
F1-F2-L3	CCGGa GGGGATGGT cTGTC	AATTGACAG ACCATCCCCt
F1-L2-F3	CCGGg GAAGTCGGT cTGTC	AATTGACAG ACCGACTTCc
L1-F2-F3	CCGGg GAAGATTGG aTGTC	AATTGACAt CCAATCTTCc
Target R (R1-R2-R3)	CCGGa GGGCGAGTC tTGTC	AATTGACAa GACTCGCCCt
F1-F2-R3	CCGGa GGGGATGGT cTGTC	AATTGACAG ACCATCCCCt
F1-R2-F3	CCGGg GAACGAGGT cTGTC	AATTGACAG ACCTCGTTCC
R1-F2-F3	CCGGg GAAGATGTC tTGTC	AATTGACAa GACATCTTCC

Fig. 6 Construction of pH3U3-target vector. (**a**) Schematic representation of construction of pH3U3-target vector. The vector can be prepared by insertion of annealed oligos into EcoRI/XmaI digested pH3U3-mcs. (**b**) Example target sequence. (**c**) List of oligos for preparation of ZFAs targeting the example sequence shown in (**b**)

7. Anneal oligos using the following conditions: 95 °C for 2 min, followed by decreasing the temperature to 25 °C, at a ramp rate of −1 °C/1.16 min.
8. Dilute extracts of pH3U3-mcs XmaI/EcoRI to 1/50. Add 1 μL of annealed oligo solution and 1 μL of diluted plasmid solution to a PCR tube. Next, add 2 μL of Ligation-Convenience Kit; gently mix. Incubate the reaction at 16 °C for 30 min. Next, transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate onto LB plates containing 30 μg/mL of kanamycin.
9. On the following day, perform colony PCR. Prepare the following master mix: 5 μL of 10× PCR buffer, 2 μL of 2 mM dNTP, 1 μL of His3-rev primer (10 μM), 1 μL of the sense oligo (10 μM) used in **step 6**, 0.25 μL of Taq polymerase, and 36.5 μL of ddH_2O.
10. Aliquot 6 μL of the master mix into each tube of an eight-strip PCR tube. To each cold PCR tube containing the PCR reaction, add a small amount of colony. Use a fine yellow pipette tip attached to a pipettor (set at 10 μL to avoid adding air to the PCR reaction), transfer the subset of colony by touching a replica plate, and pipet up and down to mix. The amount of cells should be small; just a touch will do. Sufficient mixing will result in complete cell lysis and high yields. The reaction conditions are 95 °C for 5 min; 35 cycles of 15 s at 95 °C, 15 s at 55 °C, and 20 s at 72 °C; 20 s at 72 °C; and 4 °C until sample collection.
11. Electrophorese the PCR reactions in 2% agarose gels. If the ligation was successful, you should detect a ~200 bp band.
12. Inoculate a colony with positive results into 3 mL of LB containing 30 μg/mL kanamycin; incubate overnight with agitation.
13. On the following day, purify the plasmids. Plasmids can be stored at −20 °C.
14. To confirm the success rate, we recommend preparing a negative control sample (without insert).

3.2.3 Electrocompetent Cell Preparation

1. To prepare chemically competent US0 strain, thaw glycerol stock US0 stain from deep freezer in an ice bath. Inoculate a small amount of the bacterial solution onto LB plate containing 10 μg/mL of tetracycline; incubate at 37 °C overnight.
2. On the following day, inoculate one culture tube of 3 mL of LB containing 10 μg/mL of tetracycline with 2–3 colonies from the plate, and incubate at 37 °C with agitation overnight.
3. Prepare 200 mL of SOB without antibiotics in a 500 mL flask. Aliquot 500 μL of SOB medium from the flask into a 1.5 mL

tube, for use when measuring bacterial turbidity. Add 3 mL of the overnight culture to the 500 mL flask of SOB medium, and incubate at 18 °C with agitation, until OD_{600} reaches 0.6. (It will take ~24 h to reach this OD, depending on the bacterial strain. The turbidity should be checked repeatedly.)

4. Incubate on ice bath for 20 min.
5. Transfer bacterial solution into four ice-cold 50 mL tubes. Centrifuge at 1200 × *g*, at 4 °C for 10 min. Discard the supernatant.
6. Add 16 mL each of ice-cold transformation buffer into individual tubes. Gently pipet to completely suspend the cells. Incubate on ice for 10 min.
7. Centrifuge at 1200 × *g*, at 4 °C for 10 min. Discard supernatant.
8. Add 4 mL of ice-cold transformation buffer to each tube. Gently pipet to completely suspend the cells.
9. Add 300 μL of DMSO to each tube. Because of the increment in temperature, gently mix well. Incubate 10 min on ice.
10. Aliquot 100 μL of each in to PCR tubes. Flash-freeze in liquid nitrogen; store at −80 °C.
11. Transform constructed pH3U3-target vector into a chemically competent US0 strain. Plate on LB plate with 30 μg/mL of kanamycin. On the following day, inoculate a colony into 3 mL LB medium containing 30 μg/mL of kanamycin; incubate at 37 °C with agitation (*see* **Note 3**).
12. Prepare a 200 mL flask containing 50 mL of 2× YT medium and 30 μg/mL of kanamycin. Aliquot 500 μL of 2× YT medium from the flask into a 1.5 mL tube, for use when measuring bacterial turbidity. Transfer 1 mL of bacterial medium into the 200 mL flask containing the 2× YT medium. Culture at 37 °C with agitation, until the OD_{600} reaches 0.4 (~2–3 h).
13. Transfer the culture to four 50 mL tubes; cool on ice for 20 min. For homogenous cooling, gently shake at 5 min intervals. Take care to keep the cells close to 0 °C.
14. Centrifuge for 10 min at 1200 × *g* and 4 °C. Discard supernatant.
15. Add 15 mL of ice-cold ddH_2O into each tube. Gently pipet to completely suspend the cells. Add ice-cold ddH_2O to individual tubes up to 50 mL. Centrifuge for 10 min at 1200 × *g* and 4 °C. Discard supernatant.
16. Add 15 mL of ice-cold 10% glycerol into each tube. Gently pipet to completely suspend the cells. Add ice-cold 10% glycerol to individual tubes up to 50 mL. Centrifuge at 2500 × *g*, at 4 °C for 10 min. Discard supernatant.

17. Add 1 mL of ice-cold 10% glycerol into each tube. Gently pipet to completely suspend the cells. Transfer the cell suspension to a pre-cooled 1.5 mL tube.
18. Centrifuge for 30 s at 16,000 × *g* and 4 °C. As possible, discard supernatant.
19. Add 60 μL of ice-cold 10% glycerol. Gently pipet to completely suspend the cells.
20. Flash-freeze the tube in liquid nitrogen, and store at −80 °C.

3.2.4 Electroporation

1. Thaw appropriate electrocompetent cells containing pH3U3-target vector in an ice bath. Add 1 μL of 100 ng/μL appropriate randomized library, and mix gently. Put the tube on ice for 1 min. Transfer the cells into an ice-cold 1 mm gap electroporation cuvette; perform electroporation. Recover the cells in 1 mL SOC, as described above. Incubate at 37 °C with agitation.
2. Centrifuge the cells for 10 min at 3500 × *g* and 22 °C. Discard the supernatant.
3. Add 1 mL of NM medium containing 100 μg/mL carbenicillin and 30 μg/mL kanamycin. Gently pipet to completely suspend the cells. Incubate at 37 °C for 1 h with agitation.
4. Centrifuge for 30 s at 14,000 × *g* and 22 °C. Discard supernatant.
5. Add 1 mL of ddH_2O into those tubes. Gently pipet to completely suspend the cells.
6. Centrifuge for 2 min at 14,000 × *g* and 22 °C. Discard supernatant.
7. Add 1 mL of NM medium into those tubes. Gently pipet to completely suspend the cells.
8. Centrifuge for 30 s at 14,000 × *g* and 22 °C. Discard supernatant.
9. Add 1 mL of NM medium to those tubes. Gently pipet to completely suspend the cells.

3.2.5 Plating to Large Plate

1. Using 5 μL of culture, predict the number of total transformants (*see* Subheading 3.1.4).
2. Add ~30 sterile glass beads to 3-AT selection plate (10 mM 3-AT, room temperature) and remaining bacterial solution; homogenously spread the bacteria.
3. Let the surface of the plate dry. After drying, close the lid and invert the plate. Tap the plate and let the beads drop on the lid. To prevent overdrying, seal the plate. Incubate at 37 °C for 1–3 days. Usually, 100–10,000 colonies can be obtained. Stop culturing before individual colonies merge with each other.

3.2.6 Plasmid Purification

1. Invert the plate and let the beads drop on the agarose surface.
2. Add 10 mL of 2× YT medium to the plate, and tilt to coat the surface. Wait for 2–3 min.
3. Tap the side of the plate, and suspend the cell colonies using the glass beads. Collect the cell suspension into a 15 mL tube.
4. Centrifuge for 40 s at 14,000 × *g* and 4 °C. Discard the supernatant.
5. Purify plasmids using a QIAGEN Plasmid Midi Kit.
6. Store purified plasmids at −20 °C.
7. These plasmid pools, obtained from the first selection (sublibrary), are used to construct the second selection library. These sublibraries can be used to construct other libraries.

3.3 PCR-Based In Vitro Recombination

3.3.1 Amplification of Individual ZFs

1. Amplify individual finger domains from each sublibrary, using PCR. Each finger position has a finger-specific sequence [4].
2. Amplify randomized ZF domains using the following primer set. For ZF1 amplification: ZF1-forward and ZF1-reverse; for ZF2 amplification: ZF2-forward and ZF2-reverse; for ZF3 amplification: ZF3-forward and ZF3-reverse.
3. Prepare a PCR reaction by mixing 6.8 μL of ddH$_2$O, 1 μL of Buffer for KOD -Plus-, 1 μL of 2 mM dNTP mix, 0.4 μL of 25 mM MgSO$_4$, 0.2 μL each of primers (50 μM), 0.2 μL of sublibrary plasmid, and 0.2 μL of KOD -Plus-. The reaction conditions are 94 °C for 2 min; 28 cycles of 20 s at 94 °C, 20 s at 60 °C, and 20 s at 68 °C; 20 s at 68 °C; and 4 °C until sample collection.
4. Electrophorese 5 μL of PCR reactions in 2.5% NuSieve 3:1 agarose. Excise bands of interest (ZF1: 124 bp, ZF2: 106 bp, ZF3: 126 bp). Purify DNA fragments using a QIAGEN Plasmid Midi Kit (*see* **Note 2**).

3.3.2 In Vitro Recombination

1. Construct three finger ZFs using extracted ZFs with PCR. Prepare PCR reaction master mix using 128 μL of ddH$_2$O, 20 μL of Buffer for KOD -Plus-, 20 μL of 2 mM dNTP mix, 8 μL of 25 mM MgSO$_4$, 4 μL each of primers (50 μM), 4 μL each of gel-extracted DNA solutions, and 4 μL of KOD -Plus-. Aliquot 50 μL of reaction mix into four PCR tubes. Perform PCR under the following conditions: 94 °C for 2 min; 30 cycles of 20 s at 94 °C, 1 min at 65 °C, and 1.5 min at 68 °C; 2 min at 68 °C; and 4 °C until collection of sample.
2. After PCR reaction, combine PCR products into a 1.5 mL tube. Add 20 μL of 3 M sodium acetate and 500 μL of 100% ethanol. Incubate at −80 °C for 20 min. Centrifuge at 14,000 × *g* at 4 °C for 10 min. Discard supernatant. Add

200 μL of 70% ethanol, and centrifuge at 14,000 × *g* at 4 °C for 5 min. Vacuum dry for 5 min. Dissolve in 10 μL of TE buffer, and add 2 μL of 6× loading dye (Biodynamics).

3. Electrophorese the sample in 0.8% agarose gel, containing gel indicator.
4. Excise bands with intended size (~380 bp). Purify DNA fragments using a QIAGEN Gel Extraction Kit. Extract DNA solution using 30 μL of 1/10 diluted EB buffer.
5. Digest the eluted DNA fragments with BbsI, using ~30 μL extracted DNA, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O added to 100 μL.
6. Add 10 μL of 3 M sodium acetate and 250 μL of 100% ethanol. Incubate at −80 °C for 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C. Discard supernatant. Add 200 μL of 70% ethanol, and centrifuge for 5 min at 14,000 × *g* and 4 °C. Vacuum dry for 5 min. Dissolve in 10 μL of TE buffer, and add 2 μL of 6× loading dye (Biodynamics).
7. Electrophorese the sample in 0.8% agarose gel, containing gel indicator. At this time, the DNA of interest is less abundant than in previous samples. If the band of interest is too obscure to excise, restain the gel using another DNA staining reagent (*see* **Note 2**).
8. Purify DNA fragment using QIAGEN Gel Extraction Kit. Extract DNA using 100 μL of EB buffer.
9. Add 10 μL of 3 M sodium acetate and 250 μL of 100% ethanol, and mix well. Incubate at −20 °C for 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C. Discard the supernatant. Add 70% ethanol, and centrifuge for 5 min at 14,000 × *g* and 4 °C. Discard supernatant and vacuum dry for 5 min. Dissolve in 8.5 μL of ddH_2O.

3.3.3 Ligation

1. Assemble a ligation reaction, using 2 μL of 10× ligation buffer, 1 μL of T4 DNA ligase, 1 μL of BbsI treated fragment, and 8.5 μL of pB1H2ω2+BbsI (BbsI treated, 100 ng/μL).
2. Incubate at 16 °C overnight.
3. On the following day, add 80 μL of ddH_2O and 100 μL of phenol:chloroform:isoamyl alcohol, and vigorously vortex for 30 s. Centrifuge at RT for 5 min. Transfer aqueous phase to a new 1.5 mL tube. Add 100 μL of chloroform, and vigorously vortex for 30 s. Centrifuge at RT for 5 min. Transfer aqueous phase to an ultrafilter (Amicon Ultra-0.5, 10k, Millipore), and add 400 μL of ddH_2O to fill the container.
4. Centrifuge for 10 min at 22,000 × *g* and RT. Discard filtered liquid. Add 400 μL of ddH_2O to the filter container.

Centrifuge at 22,000 × *g* at RT, until ~20 μL of unfiltered liquid remains. Store unfiltered liquid (DNA solution) at −20 °C.

3.4 Second B1H Selection

3.4.1 Construction of pH3U3-Target Vector

1. Prepare pH3U3 vector for the second selection, as described before (*see* Subheading 3.2.2).

3.4.2 Preparation of Electrocompetent US0 (pH3U3) Cells

1. Prepare electrocompetent US0*ΔhisBΔpyrFΔrpoZ* transformed with appropriate pH3U3 vector (*see* Subheading 3.2.3).

3.4.3 Electroporation and Plating to Large Plates

1. Perform electroporation as described in Subheadings 3.2.3, 3.2.4, and 3.2.5. However, use ultrafiltered 5 μL DNA solution for electroporation. Furthermore, adjust the 3-AT concentration to 20 mM for large plates.
2. Usually, transformation efficiency is 10^5–10^6.
3. Culture the plate at 37 °C for 1–3 days until colonies grow large enough to pick. If the number of colonies is lower than ten, the chance of obtaining functional ZFNs is quite low.

3.4.4 Colony PCR

1. Perform colony PCR. Prepare a master mix, using 42.35 μL of ddH_2O, 5 μL of 10× PCR buffer, 2 μL of 2 mM dNTP, 0.2 μL of pB1H2ω2-seq-F (50 μM), 0.2 μL of pB1H2ω2-seq-R (50 μM), and 0.25 μL of Taq polymerase.
2. Aliquot 6 μL of the reaction mixture into each tube of an eight-strip PCR tube. To each cold PCR tube containing the PCR reaction, add a small amount of colony. To perform these inoculations, use a fine yellow pipette tip attached to a pipettor (set at 10 μL to avoid addition of air into the PCR reaction); transfer the colony subset by touching a replica plate, and pipetting up and down to mix. The amount of cells should be small; just a touch will do. Sufficient mixing will result in complete cell lysis and high yields. Pick colonies in descending order of size. Perform PCR under the following conditions: 95 °C for 5 min; 30 cycles of 15 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C; 20 s at 72 °C; and 4 °C until collection of sample.
3. Digest unpurified PCR product by BsgI and XbaI, using 5.8 μL of ddH_2O, 1 μL of CutSmart buffer, 1 μL of 1/40 diluted SAM (supplied with BsgI from NEB), 2 μL of PCR reaction, 0.1 μL of XbaI, and 0.1 μL of BsgI.
4. Incubate at 37 °C for 3 h. Electrophorese in 2.5% NuSieve 3:1 agarose gel. Excise about 256 bp bands.

5. Digest pSTL plasmid by BsgI, using 13.6 μL of ddH_2O, 2 μL of CutSmart buffer, 2 μL of 1/40 diluted SAM (supplied with BsgI from NEB), 4 μL of pSLT (200 ng/μL), and 0.4 μL of BsgI.
6. Incubate at 37 °C overnight. Purify DNA fragments using QIAGEN PCR Purification Kit. Elute DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
7. Digest purified pSTL plasmid by XbaI, using 2 μL of CutSmart buffer, 17.6 μL of purified pSLT solution, and 0.4 μL of BsgI.
8. Incubate at 37 °C at least for 3 h. Next, add 1 μL of rAPid alkaline phosphatase, and mix well. Incubate at 37 °C at least for 30 min.
9. Electrophorese in 1% agarose gel. Excise about 5.6 kbp bands.
10. Purify the DNA fragments using QIAGEN Gel Extraction Kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
11. Add 1 μL of ZFA fragment solution and 1 μL of pSTL plasmid solution into a PCR tube. Add 2 μL of Ligation-Convenience Kit, and gently mix. Incubate the reaction at 16 °C for 30 min. Next, transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate them onto an LB plate containing 100 μg/mL ampicillin.
12. On the following day, perform colony PCR (*see* Subheading 3.4.4). Use T7 promoter primer and S418-R primer.
13. Electrophorese the PCR reactions in 2% agarose gels. If ligation was successful, you should detect a 479 bp band.
14. Inoculate a colony with positive results into 3 mL of LB containing 30 μg/mL kanamycin; incubate overnight with agitation.
15. On the next day, purify transfection grade plasmids using an appropriate kit.
16. Add 10 μL of 3 M sodium acetate (per 100 μL starting material), and mix well. Next, add 110 μL of phenol:chloroform: isoamyl alcohol and vortex for 30 s. Centrifuge 14,000 × *g* at room temperature for 5 min.
17. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 110 μL of chloroform and vortex for 30 s. Centrifuge at 14,000 × *g* at room temperature for 5 min.
18. Repeat **step 4**.
19. Carefully transfer the aqueous phase to a new 1.5 mL tube. Add 250 μL of 100% ethanol and mix well. Incubate at −20 °C for more than 20 min. Centrifuge at 14,000 × *g*, at 4 °C for 10 min.

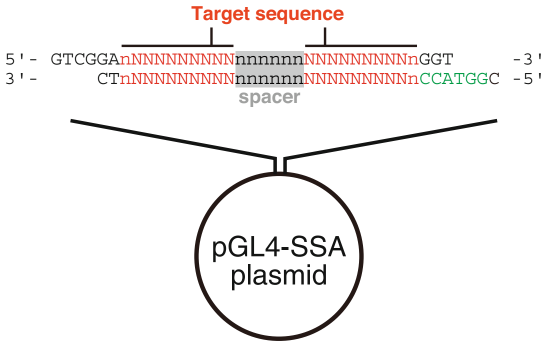

Target sequence
5'- GTCGGAnNNNNNNNNNnnnnnnNNNNNNNNNNnGGT -3'
3'- CTnNNNNNNNNNnnnnnnNNNNNNNNNNnCCATGGC -5'
spacer
pGL4-SSA plasmid

Fig. 7 Construction of pGL4-SSA-target vector. Schematic representation of construction of pGL4-SSA-target vector. Prepare appropriate oligos containing ZFN target sites, and additional adaptor sequence. Green letters represent KpnI site that is generated only after insertion of annealed oligos

20. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.
21. Dry the pellet in vacuum for 5 min. Dissolve it in 20 μL of ddH_2O. Adjust the concentration to 300 ng/μL. Plasmids can be stored at −20 °C.

3.5 SSA Assay

3.5.1 Construction of pGL4-SSA

1. Prepare oligos containing target sequence, as described in Fig. 7.
2. Digest purified pGL4-SSA plasmid by BsaI, using 1 μL of CutSmart buffer, 2 μL of pGL4-SSA (200 ng/μL), and 0.4 μL of BsaI.
3. Incubate at 37 °C at least for 3 h. Next, add 1 μL of rAPid alkaline phosphatase, and mix well. Incubate at 37 °C, for at least 30 min.
4. Electrophorese in 1% agarose gel. Excise about 5.6 kbp bands.
5. Purify the DNA fragments using QIAGEN Gel Extraction Kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
6. Prepare reaction mixture, using 1 μL of 10× annealing buffer, 1 μL each of oligos prepared in **step 1** (50 μM), and 7 μL of ddH_2O.
7. Anneal oligos using the following conditions: 95 °C for 2 min, followed by decreasing temperature to 25 °C at a ramp rate of −1 °C/1.16 min.
8. Add 1 μL of annealed oligo solution and 1 μL of purified plasmid solution to a PCR tube. Add 2 μL of Ligation-Convenience Kit, and gently mix. Incubate the reaction at 16 °C for 30 min. Transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate onto an LB plate containing 30 μg/mL kanamycin.

Table 2
Mixture of reagents for SSA assay

	Negative control (μL)	Positive control (μL)	Sample (μL)
pSTL-ZFN (300 ng/μL)	–	–	0.66
pSTL-ZFA36 (300 ng/μL)	–	0.66	–
pGL4-SSA-ZFN target (150 ng/μL)	0.66	0.66	0.66
pRL-CMV (30 ng/μL)	0.66	0.66	0.66
Opti-MEM	23	23	23

9. On the following day, inoculate 2–3 colonies into separate tubes with 3 mL LB medium containing 30 μg/mL kanamycin; incubate overnight with agitation.
10. On the following day, purify the plasmids using an appropriate kit. Digest purified plasmids by KpnI as follows: 1 μL of CutSmart buffer, 2 μL of plasmid (200 ng/μL), and 0.4 μL of KpnI.
11. Incubate at 37 °C at least for 3 h. Next, electrophorese all reactions on a 1% agarose gel. After electrophoresis and appropriate DNA dye staining, check the DNA fragment size. If appropriately constructed, 3800 and 1800 bp bands should be visible. Plasmids can be stored at −20 °C.

3.5.2 Luc Assay

1. Culture HEK293T cells in growth medium containing 10% FBS.
2. Prepare the mixture described in Table 2 in wells of a 96-well plate.
3. Dilute 0.7 μL of Lipofectamine LTX with 25 μL of Opti-MEM (prepare master mix); add this mixture to the DNA-Opti-MEM containing wells within 5 min. Incubate at room temperature for 30 min.
4. Prepare HEK293T cell suspension at 5×10^4 cells/100 μL in growth medium by standard trypsinization protocol. Next, add 100 μL of cell suspension to DNA-Lipofectamine mixture-containing wells. Mix by pipetting.
5. Incubate for 24 h at 5% CO_2 and 37 °C.
6. Discard 75 μL each of medium from cultures.
7. Add 75 μL of Dual-Glo Luciferase Reagent (Dual-Glo luciferase assay system; Promega). Incubate at room temperature for 10 min. Transfer all samples to a white 96-well plate.
8. Quantify luciferase activities using a plate reader.

9. Add 75 μL of Dual-Glo Stop & Glo reagent (Dual-Glo luciferase assay system; Promega). Incubate at room temperature for 10 min.
10. Quantify luciferase activities using the plate reader.

4 Notes

1. Although 20 mM uracil and adenine stock solutions can be prepared, precipitate might appear when they are stored at 4 °C. These precipitates can be removed by incubation in a hot bath. Preparation of medium using 20 mM uracil and/or adenine stocks will not work appropriately.
2. If gels are stained with DNA-binding fluorescent dye, dye excitation should be minimized as much as possible.
3. It is important that all bacteria possess pH3U3 vector. Therefore, avoid overgrowth by transferring the culture plate to room temperature until inoculating a starter culture. Do not store the culture plate at 4 °C.

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We thank Dr. Scot Wolfe for providing the pH3U3-mcs reporter vector and US0Δ*hisB*Δ*pyrF*Δ*rpoZ* bacterial strain (Addgene plasmids 12609 and 18049, respectively). This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (15K18467, 16H01407) and by the JST PRESTO program to H.O.

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Chapter 2

Updated Overview of TALEN Construction Systems

Tetsushi Sakuma and Takashi Yamamoto

Abstract

Transcription activator-like effector (TALE) nuclease (TALEN) is the second-generation genome editing tool consisting of TALE protein containing customizable DNA-binding repeats and nuclease domain of FokI enzyme. Each DNA-binding repeat recognizes one base of double-strand DNA, and functional TALEN can be created by a simple modular assembly of these repeats. To easily and efficiently assemble the highly repetitive DNA-binding repeat arrays, various construction systems such as Golden Gate assembly, serial ligation, and ligation-independent cloning have been reported. In this chapter, we summarize the updated situation of these systems and publicly available reagents and protocols, enabling optimal selection of best suited systems for every researcher who wants to utilize TALENs in various research fields.

Key words TALEN, Golden Gate assembly, Pre-assembled library, Serial ligation, Ligation-independent cloning

1 Introduction

The rapid emergence of clustered regularly short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) caused a paradigm shift in genome editing study [1]; however, TALEN is still an important genetic tool for functional genomics study, because various characteristics of these two tools differ from each other [2]. For example, CRISPR-Cas9 normally works as a monomer nuclease, while TALEN works as a dimer. CRISPR-Cas9 produces blunt ends, while TALEN produces cohesive ends. CRISPR-Cas9 consists of a complex of protein and RNA, while TALEN consists of protein only. These differences enable complementary usage of these two technologies.

Functional TALEN protein can be created by the assembly of DNA-binding repeats, in which the specificity of base recognition is defined by repeat-variable di-residue (RVD) [3, 4], into the backbone of TALEN harboring N- and C-terminal domains. Each single repeat consists of 34 amino acids, where the 12th and 13th residues are called RVD. Amino acid composition other than RVD

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among repeats is basically the same, but some non-RVD variations on particular positions such as 4th and 32nd residues have been determined to be important for TALEN activity [5, 6]. The lengths and amino acid sequence of N- and C-terminal domains of TALE are also identified as important factors for the activity and specificity of TALENs [7–9]. In addition, other contexts such as promoter, UTRs, poly-A signal sequence, and codon usage of TALEN-coding sequence are also important for the appropriate use in various cells and organisms. Therefore, proper choice of TALEN structure and vector backbone is needed in terms of the best practical application as well as the selection of construction systems described below.

Typically, 15–20 repeats have to be assembled in a defined order to construct functional TALENs. To achieve this, various one- or two-step modular assembly methods using single or multiple repeat libraries have been developed, improved, and applied by many research groups. In this chapter, we describe an overview of the current construction systems of TALEN, providing brief introduction of each system and detailed information of publicly available plasmid kits and protocols.

2 Summary of TALEN Construction Kits

Generally, the custom-made TALEN is created using a set of plasmids packaged for its construction. Such plasmid kits can be obtained from Addgene, the nonprofit plasmid repository [10]. Table 1 summarizes a current list of TALEN kits available from Addgene. There are various differences among these kits, including the method of modular assembly, the number of plasmids, and framework of TALEN scaffold. Regarding the construction method, a one-pot cloning strategy called Golden Gate assembly is most widely used. This method enables the simultaneous digestion and ligation of multiple plasmids in a single tube with high efficiency and accuracy, without requiring any special equipment other than a standard thermal cycler. Thus, the current TALEN kits are mostly based on this method. Other methods, however, are adopted in some kits, and they have unique characteristics that might be advantageous for a particular use. The Joung Lab TAL Effector Engineering Reagents are for the simple restriction digestion and ligation of modules in a serial manner, which can commonly be utilized for a low-/middle-/high-throughput generation of custom TALENs. The LIC TAL Effector Assembly Kit is based on a ligation-independent cloning (LIC) method, which does not require a recombinant ligase, but depends on the annealing of relatively long overhangs. The following sections are the detailed information of these methods and systems to provide the best understanding.

Table 1
Summary of TALEN kits available from Addgene

Kit name	Kit ID	Depositor	Assembly method	Number of plasmids	Repeat number
Golden Gate TALEN and TAL Effector Kit 2.0	1000000024	Daniel Voytas and Adam Bogdanove	Golden Gate	86	12–31
Platinum Gate TALEN Kit	1000000043	Takashi Yamamoto	Golden Gate	35	6–21
TALE Toolbox	1000000019	Feng Zhang	PCR/Golden Gate	12	13, 19, 25
Musunuru/Cowan Lab TALEN Kit	1000000034	Kiran Musunuru and Chad Cowan	Golden Gate	834	15
Ekker Lab TALEN Kit	1000000038	Stephen Ekker	Golden Gate	256	15
FusX TALEN assembly system	1000000063	Stephen Ekker	Golden Gate	336	15, 16
REAL Assembly TALEN Kit	1000000017	Keith Joung	Serial ligation	32	Any number
LIC TAL Effector Assembly Kit	1000000023	Veit Hornung	Ligation-independent cloning	76	10–19

3 Golden Gate Assembly-Based Systems

3.1 Introduction of Golden Gate Assembly

Golden Gate assembly was first reported in 2008 [11], and soon after the initial publication, the high capacity of simultaneous assembly of many fragments (nine inserts in the acceptor vector) was shown [12]. In this assembly method, type IIS restriction enzymes such as BsaI and BsmBI are used to generate various patterns of cohesive ends at the same time by using a single enzyme, enabling simultaneous ligation of multiple modules in a defined order (Fig. 1). The recognition sequence of the enzyme on the insert plasmid is placed outside the module sequence, while that on the acceptor vector is placed inside the vector backbone sequence. It results in not only a seamless cloning but also a prevention of re-cutting the assembled products. In addition, a lacZ cassette for the blue/white selection in the cut-out region of the acceptor vector and different antibiotic selection marker on the insert vectors can reduce the false-positive clones. Thus, the Golden Gate assembly method is a very sophisticated protocol for tandem ligation of multiple modules, which is quite suited for the TALE repeat assembly.

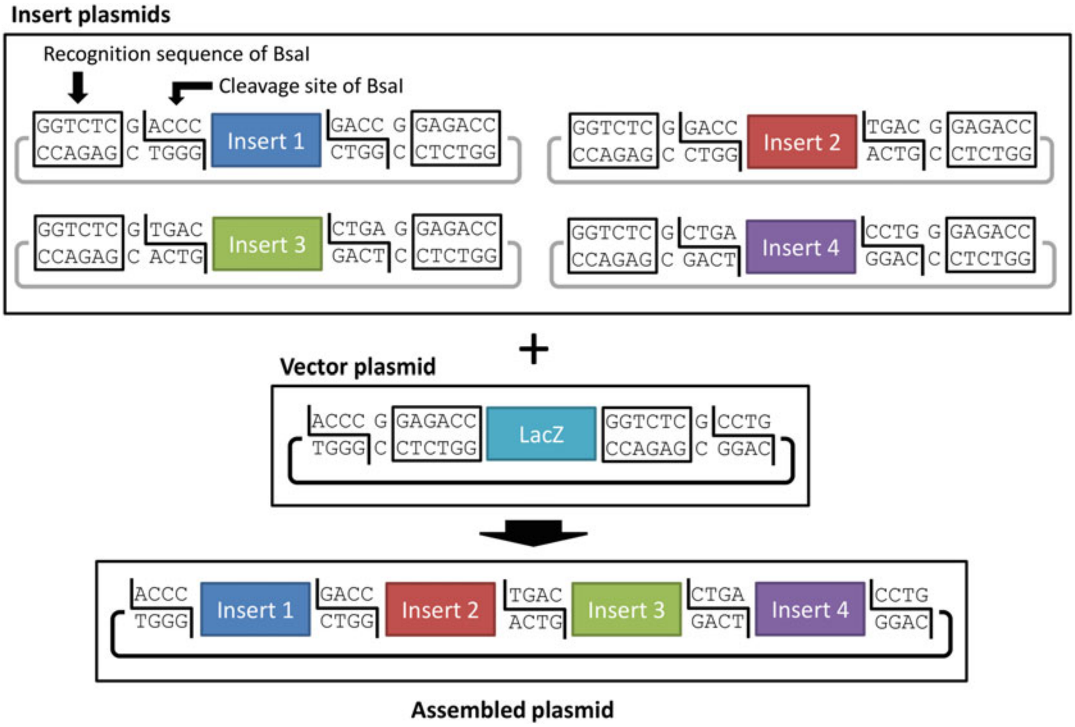
Insert plasmids
Recognition sequence of BsaI
Cleavage site of BsaI
GGTCTC G ACCC / CCAGAG C TGGG
Insert 1
GACC G GAGACC / CTGG C CTCTGG
GGTCTC G GACC / CCAGAG C CTGG
Insert 2
TGAC G GAGACC / ACTG C CTCTGG
GGTCTC G TGAC / CCAGAG C ACTG
Insert 3
CTGA G GAGACC / GACT C CTCTGG
GGTCTC G CTGA / CCAGAG C GACT
Insert 4
CCTG G GAGACC / GGAC C CTCTGG
+
Vector plasmid
ACCC G GAGACC / TGGG C CTCTGG
LacZ
GGTCTC G CCTG / CCAGAG C GGAC
ACCC / TGGG
Insert 1
GACC / CTGG
Insert 2
TGAC / ACTG
Insert 3
CTGA / GACT
Insert 4
CCTG / GGAC
Assembled plasmid

Fig. 1 Example of Golden Gate assembly. The assembly of four inserts mediated by BsaI enzyme is shown. The four inserts are tandemly ligated into the vector plasmid without carrying BsaI recognition sequences

3.2 Typical Protocol for the Golden Gate Assembly-Based TALEN Construction

1. Prepare all the plasmids needed and design TALEN target sequences.
2. Mix the insert and vector plasmids with a restriction enzyme and a DNA ligase in a single PCR tube, and perform first-step Golden Gate assembly in a thermal cycler. Typically, up to ten modules are tandemly assembled in one intermediate array vector.
3. Perform bacterial transformation with blue/white selection. Screen the intended clones by colony PCR. Culture the clones and extract the plasmid DNA.
4. Perform second-step Golden Gate assembly, transformation, colony screening, and plasmid preparation in a similar way. All the procedures can be conducted in 5 days [13, 14].

3.3 Original Golden Gate Kit from Voytas/Bogdanove Lab

The first TALEN kit appeared in Addgene is the Golden Gate TALEN and TAL Effector Kit, deposited by Daniel Voytas and Adam Bogdanove [13]. Currently, the version 2.0 of the kit is available (Kit #1000000024). The kit contains 86 plasmids, including 60 module vectors, 5 last repeat vectors, 13 intermediate array vectors, and 6 destination vectors to create TALEN vectors containing 12–31 DNA-binding repeats with no non-RVD variations

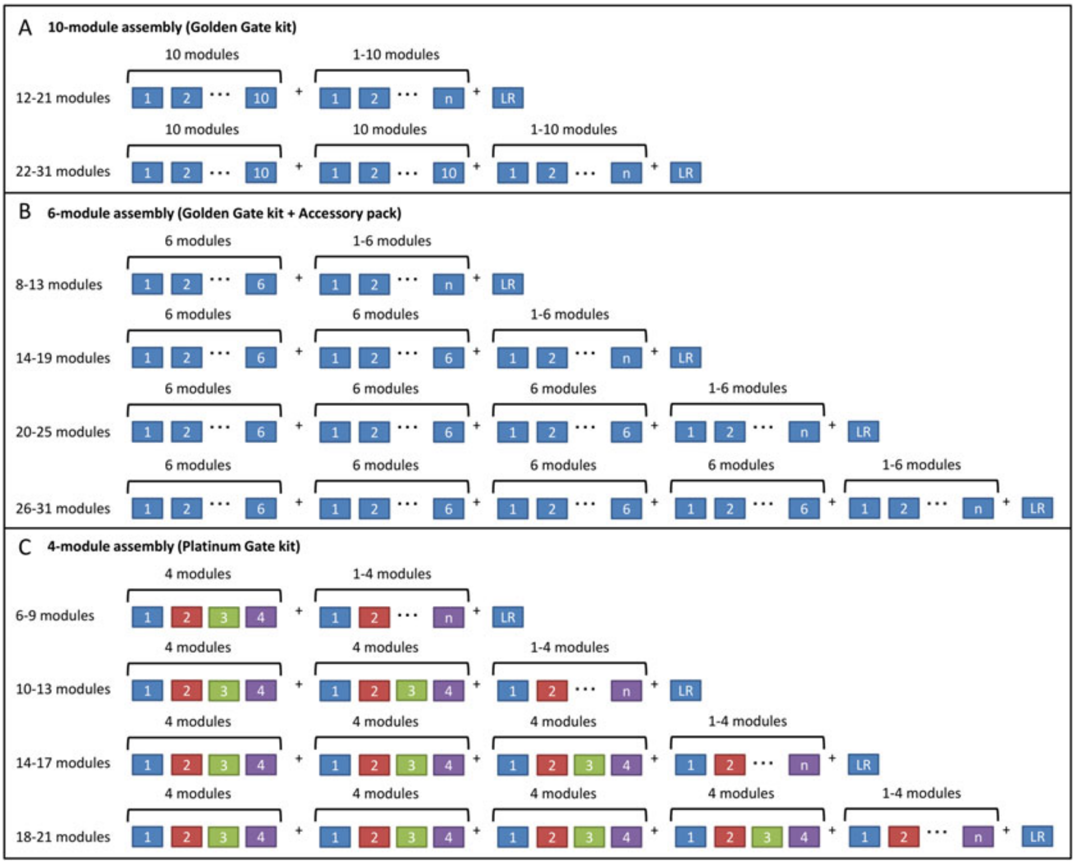

Fig. 2 Summary of ten-, six-, and four-module assembly needed for the Golden Gate Kit (**a**), the Golden Gate Kit with the accessory pack (**b**), and the Platinum Gate Kit (**c**), respectively. Up to 31 (**a**, **b**) or 21 (**c**) modules can be assembled with the two-step Golden Gate assembly systems. In the systems based on the Golden Gate Kit, all the DNA-binding modules consist of the same amino acid composition excluding RVDs (**a**, **b**), whereas those in the TALENs created with the Platinum Gate Kit consist of variable repeats (**c**). See the main text for details. LR, last repeat

(Fig. 2a). The functionality of the TALEN vector created using this kit has been widely validated in many cells and organisms; however, the vector often cannot be used directly and needs additional plasmids, because the original kit only contains yeast expression vectors as destination vectors. In addition, the reaction of ten-module assembly is relatively difficult and not always successful. In this regard, the add-on plasmids compatible with the Golden Gate kit have been created and deposited in Addgene by many groups.

3.4 Accessory Plasmids for the Golden Gate Kit

To make the most of the Golden Gate kit, various add-on plasmids have been developed (Table 2). All of them are destination vectors and distributed as single plasmids, excluding the TALEN Construction Accessory Pack (Kit #1000000030), developed by our

Table 2
Summary of Golden Gate add-ons

Kit/plasmid name	Kit/plasmid ID	Depositor	Contents	Number of plasmids
TALEN Construction Accessory Pack	1000000030	Takashi Yamamoto	Intermediate array vectors and destination vectors for in vitro transcription and mammalian expression	9
RCIscript-GoldyTALEN and pC-GoldyTALEN	38142, 38143	Daniel Carlson	Destination vectors for in vitro transcription and expression of TALENs	2
TAL5-BB and pTAL6-BB	36033, 36034	Tom Ellis	Destination vectors for yeast expression of TALE-based transcriptional repressors	2
pCS2TAL3-DD and pCS2TALE3-RR	37275, 37276, 48636, 48637	David Grunwald	Destination vectors for in vitro transcription and expression of TALENs	4
pCAG-T7-TALEN(Sangamo)-Destination Constructs	37184, 40131, 40132	Pawel Pelczar	Destination vectors for in vitro transcription and expression of TALENs	3
pcDNA3.1-GoldenGate and pcDNA3.1-GoldenGate-VP64	47388, 47389	Charles Gersbach	Destination vectors for mammalian expression of TALE-based transcriptional activators	2
pTALYM3 and pTALYM4	47874, 47875	Maria-Elena Torres-Padilla	Destination vectors for mammalian expression of fluorescent TALE to visualize chromosome dynamics	2
pTAL7a and pTAL7b	48705, 48706	Boris Greber	Destination vectors for the application of TALEN technology in human pluripotent stem cells	2
pBlue-TAL	49401	Michal Zurovec	Destination vector for the generation of germline mutations in *Bombyx mori* and *Drosophila melanogaster*	1

group [14]. This accessory pack contains nine plasmids including seven intermediate array vectors and two destination vectors. The unique intermediate array vectors are, of course, compatible with the Golden Gate kit, and they enable six-module assembly, which results in efficient and accurate concatemerization of the TALE repeats without any additional cloning steps (Fig. 2b). The destination vectors are for in vitro transcription using T7 RNA polymerase and expression in mammalian cells via CMV or CAG promoters, harboring truncated TALEN scaffolds with enhanced activity and specificity (47 amino acids for C-terminal domain) [8, 14].

Regarding other plasmids, RCIscript-GoldyTALEN and pC-GoldyTALEN (Plasmid #38142 and #38143), pCS2TAL3-DD and pCS2TALE3-RR (Plasmid #37275, #37276, #48636, and #48637), and pCAG-T7-TALEN(Sangamo)-Destination Constructs (Plasmid #37184, #40131, and #40132) are in vitro transcription and expression vectors with CMV or CAG promoters for TALENs. TAL5-BB and pTAL6-BB (Plasmid #36033 and #36034) are yeast expression vectors for TALE-based transcriptional repression. pcDNA3.1-GoldenGate and pcDNA3.1-GoldenGate-VP64 (Plasmid #47388 and #47389) are mammalian expression/in vitro transcription vector for TALE expression and TALE-based transcriptional activation, respectively. pTALYM3 and pTALYM4 (Plasmid #47874 and #47875) are mammalian expression vectors to visualize the dynamics of the chromosome using the systems named TALE-mediated Genome Visualization (TGV) [15]. pTAL7a and pTAL7b (Plasmid #48705 and #48706) are destination vectors for the TALEN application in human pluripotent stem cells. pBlue-TAL (Plasmid #49401) is for the generation of germline mutations in *Bombyx mori* and *Drosophila melanogaster*.

3.5 Platinum Gate Kit from Yamamoto Lab

Although the convenience of the Golden Gate kit has become a higher level by utilizing various additional plasmids, the activity of resultant TALENs is not always sufficient, requiring improvement of amino acid composition of the TALE repeats. We previously found that periodically patterned variable repeats can enhance the DSB-inducing activity of TALENs [5, 15]. The Platinum Gate TALEN kit (Kit #100000004) is the only one distributed via Addgene that highly active TALENs with the variable repeats can be created using Golden Gate assembly method. The versatility of TALENs constructed with this kit, referred to as Platinum TALENs, has been proven in a number of publications, reporting various applications in a wide variety of cells and organisms, such as human iPS cells [16, 17], fungi [18, 19], microalgae [20, 21], plants [22], nematodes [23], sea urchins [24], ascidians [25], water flea [26], zebrafish [27], medaka fish [28], tuna fish [29, 30], newts [31], frogs [5], mice [32], rats [5], pigs [33], and marmosets [34]. In addition, similar to the original Golden Gate Kit, the Platinum TALE architecture can also be utilized for TALE-based transcriptional control and epigenome editing [35, 36].

The kit contains 35 plasmids including 16 module vectors, 11 intermediate array vectors, and 8 destination vectors. TALENs with 6–21 repeats can be constructed using 4-module assembly method (Fig. 2c). Fewer numbers of assembled modules compared with previous systems (4 vs. 10 or 6) result in an easy, robust, and efficient assembly reaction. Destination vectors are designed to be applicable in mammalian expression and in vitro transcription, similar to the previous ones. Moreover, two truncation patterns of N- and C-terminal domains of TALE based on different TALE architecture (+153/+47 of PthXo1 and +136/+63 of AvrBs) are included. These two scaffolds have different preferences on the TALEN activity; +153/+47 TALEN is active with limited spacer lengths, while +136/+63 TALEN is active with broad spacer lengths [5]. Users can choose the appropriate scaffold from these two options depending on the intended use. The detailed protocol to construct Platinum TALEN was described in a previous publication [37].

3.6 TALE Toolbox from Zhang Lab (Combined Method of Golden Gate Assembly and PCR Amplification)

Another popular TALEN kit based on Golden Gate assembly is the TALE Toolbox (Kit #1000000019) developed by Feng Zhang [38]. The TALE Toolbox consists of a relatively small number of plasmids: four module vectors and eight destination vectors. Among them, four destination vectors are for TALE-based transcriptional activation; thus, only eight plasmids are needed to create TALENs. However, this kit requires several additional procedures including PCR amplification, purification of PCR products, exonuclease treatment, another round of PCR amplification, and gel extraction and purification. Therefore, although the initial cost and labor is low compared with the other methods, users have to consider the running cost and labor. Two rounds of PCR amplifications might introduce unintended errors. Variable repeat TALENs cannot be created, because there are only four module vectors.

As the add-on plasmids for the TALE Toolbox, TALEColor plasmids are available via Addgene (Plasmids #49622–#49647). TALEColor is a system to fluorescently label the specific repetitive sequence in the genome [39].

3.7 Multiple Module Library-Mediated Systems from Ekker Lab and Musunuru/Cowan Lab

As described so far, Golden Gate assembly-based construction of TALENs basically depends on the two-step assembly process. However, there are several systems enabling one-step construction of TALEN vectors by using pre-assembled module libraries. Musunuru/Cowan Lab TALEN Kit (Kit #1000000034) contains 834 plasmids including 58 three-module library, 768 four-module library, and 2 destination vectors (pTAL_GFP and pTAL_RFP) [40]. Using these large-scale libraries, 15-repeat TALENs can be constructed with a single cloning step. Stephen Ekker lab first deposited the partial library containing 256 4-module plasmid sets (Ekker Lab TALEN Kit; Kit #1000000038), which is compatible with the Golden Gate Kit [41]. This plasmid set can help in creating 15-repeat TALENs, but requires additional construction of intermediate array plasmids. Subsequently, Ekker lab has developed another system called FusX TALEN Assembly System (Kit #1000000063) [42]. The FusX system contains 336 plasmids, enabling 1-step construction of TALENs without additional intermediate constructs. This system is also compatible with the Golden Gate Kits; thus, all the accessory plasmids for the Golden Gate Kit can also be used. These multiple module library-mediated methods are suitable for high-throughput production of TALEN vectors, while there also are some limitations. First, hundreds of pre-assembled plasmids are needed and have to be maintained. Second, assemblable repeat length is very limited (15 repeats in the Musunuru/Cowan system and 15 or 16 repeats in FusX system). Since the N-terminal domain of TALE recognizes a thymine, this limitation narrows the target range of TALENs. Third, incorporation of repeat variations such as variable repeats and noncanonical RVD repeats [43] is very difficult.

4 Other Systems

4.1 TAL Effector Engineering Reagents from Joung Lab (Serial Ligation Method)

Keith Joung lab has provided the REAL Assembly TALEN Kit (Kit #1000000017) that consists of 32 plasmids including 28 module vectors and 4 destination vectors [44]. Since the REAL assembly method depends on the standard serial digestion and ligation procedure, it requires several cloning steps to create functional TALENs. To improve the throughput, the pre-assembled module library can be used, similar to the Golden Gate assembly-based systems (REAL-Fast method). The detailed procedures for creating TALENs using REAL or REAL-Fast method were described in a previous publication [45]. In addition, the fast ligation-based automatable solid-phase high-throughput (FLASH) method can also be applicable with the same reagents [46, 47]. The FLASH system enables automated or manual TALEN assembly in a 96-well format. Moreover, TALENs constructed with these systems contain variable repeats similar to the Platinum TALENs. However, the pre-assembled module library for the REAL-Fast and FLASH systems has not yet distributed via Addgene.

Similar to other systems, there are several vectors compatible with the REAL Assembly TALEN Kit available via Addgene. TALE transcriptional activator expression vectors (Plasmids #43884–#43891) are the destination vectors to express TALE protein fused with a VP64 or NF-κB p65 transcriptional activation domain [48]. EMM65, EMM67, and EMM71T (Plasmids #49042–#49044) are the destination vectors to express TALE protein fused with an LSD1 histone demethylase [49].

4.2 LIC Assembly Kit from Hornung Lab (Ligation-Independent Cloning Method)

Veit Hornung lab has reported another approach for TALEN assembly. They developed a ligation-independent cloning (LIC) assembly-mediated system (LIC TAL Effector Assembly Kit; Kit #1000000023) [50]. In this system, a unique procedure named chew back reaction is required. The chew back reaction is a programmed DNA end resection mediated by T4 DNA polymerase, resulting in unique 5′ overhangs for the annealing of multiple DNA fragments in a defined order. The kit contains 76 plasmids, creating 10- to 19-repeat TALENs with two assembly steps. The detailed procedures for LIC-based TALEN construction were described in a previous publication [51].

4.3 Other Systems Currently Unavailable from Addgene

Besides the Addgene kits, there are a number of alternative TALEN construction systems reported, such as iterative capped assembly (ICA) system [52], uracil-specific excision reagent (USER) cloning-based methods [53, 54], STAR method based on isothermal assembly [55], and synthetic oligonucleotide-mediated system [56]. It is highly desired for these interesting systems to be available via Addgene in the future.

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Chapter 3

CRISPR/Cas9

Izuho Hatada, Sumiyo Morita, and Takuro Horii

Abstract

CRISPR/Cas9 is the genome-editing technology that is most widely used around the world. Its widespread adoption is largely due to its simplicity and ease of use. Here, we introduce the construction of vectors and genome editing of the target gene in cells using the CRISPR/Cas9 system.

Key words CRISPR/Cas9, Cas9, gRNA

1 Introduction

Clustered regularly at interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease 9 (CRISPR/Cas9) is a relatively simple genome-editing technology that has become widely adopted around the world [1]. The method requires only Cas9 nuclease and a short RNA, called single-guide RNA (gRNA or sgRNA), which includes 20 nucleotides complementary to the target sequence in the genome; when present, these two factors induce cleavage of the target. Earlier genome-editing systems, such as zinc finger nucleases (ZFNs) [2] and transcription activator-like effector nucleases (TALENs) [3], require complicated processes for construction of DNA-binding proteins, making these methods time-consuming and expensive. Thus, CRISPR/Cas9 is the most widely used genome-editing technology.

For the design of gRNA, the only restriction is that the locus needs to contain the "NGG" protospacer adjacent motif (PAM) downstream of the target sequence (Fig. 1) [1]. Therefore, the user only needs to design the gRNA and construct the gRNA expression vector. After transfection of cells with the gRNA and Cas9 expression vectors, the Cas9/gRNA complex binds to the target genomic locus and induces double-strand breaks (DSBs) (Fig. 1). Cas9-induced DSBs can be repaired by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ-mediated

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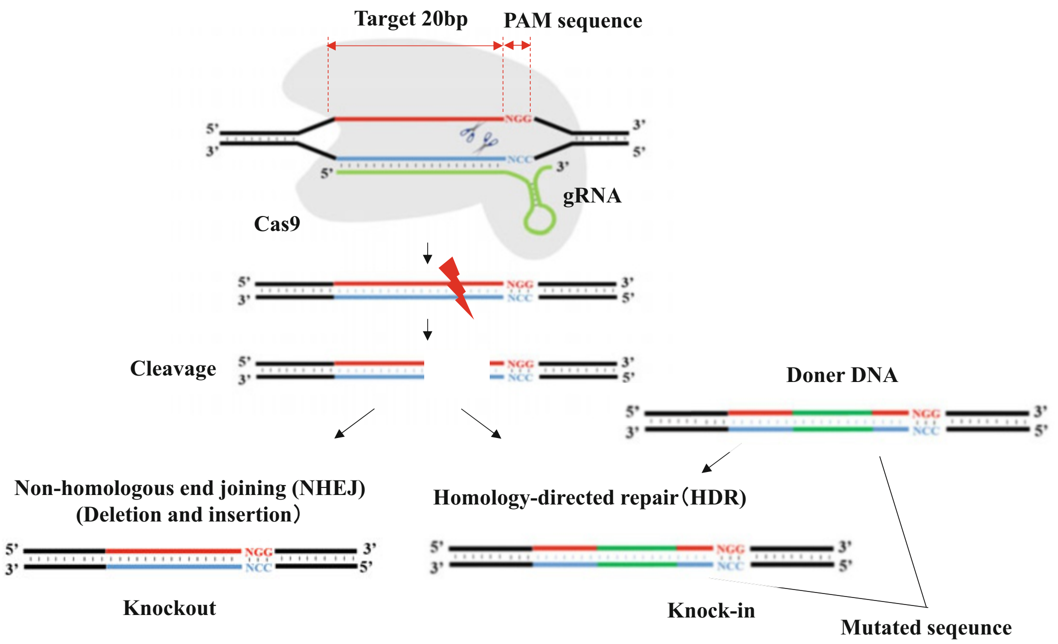

Fig. 1 Schematic of the CRISPR/Cas9 system. The Cas9 endonuclease and gRNA complex cleave the target sequence. The PAM (NGG) adjacent to the 3′ end of the 20 bp target sequence is essential for cleavage. The cleaved target is frequently repaired by spontaneous non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ results in a small deletion or insertion at the targeted locus, whereas HDR generates a knock-in at the targeted locus if donor DNA is present

repair usually leads to small insertions or deletions (indels) at the targeted site, leading to frameshift mutations (Fig. 1). By contrast, in the presence of a single- or double-stranded DNA template containing homology to the sequences flanking the DSB, HDR can lead to precise point mutations or DNA insertions (Fig. 1).

Here, we introduce the construction of gRNA vectors and genome editing of target sequences in cells, as well as the validation of editing efficiency.

2 Materials

2.1 sgRNA Design

1. CRISPRdirect: http://crispr.dbcls.jp/ [4].
2. UCSC Genome Browser: https://genome.ucsc.edu/cgi-bin/hgGateway.

2.2 Preparation of Cassette DNA

1. 10 × PCR buffer: 100 mM Tris–HCl (pH 8.9), 500 mM KCl, 15 mM $MgCl_2$.
2. XgRNA-S (100 pmol/μL) oligonucleotide described in Subheading 3.1.
3. XgRNA-AS (100 pmol/μL) oligonucleotide described in Subheading 3.1.

2.3 Preparation of Vector

1. gRNA_Cloning Vector BbsI: an empty gRNA expression vector, used to create a gRNA targeting a specific sequence (#128433, Addgene).
2. Restriction enzyme BbsI.
3. Gel extraction kit such as QIAquick Gel Extraction Kit (#28704, QIAGEN).

2.4 Ligation

1. T4 DNA ligase.

2.5 Transformation and Confirmation of the Clone

1. *Escherichia coli* competent cells.
2. LB agar with kanamycin: 1.6% Agar, 1% tryptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL kanamycin (add 10 mg/mL stock solution after cooling down the autoclaved mix).
3. DNA sequencing kit.
4. Capillary DNA sequencer.
5. LB with kanamycin: 1% tryptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL kanamycin (add 10 mg/mL stock solution after cooling down the autoclaved mix).
6. Plasmid DNA purification kit.
7. Thermal cycler.
8. PCR reagents.
9. Universal M13 Forward (−20) primer: GTAAAACGA CGGCCAG.
10. Universal M13 Reverse primer: CAGGAAACAGCTATGAC.

2.6 Validation of gRNA

1. Lipofection reagent such as Lipofectamine 2000 (Thermo Fisher Scientific).
2. Cells for transfection and its culture medium. We usually use mouse ES cells for mouse gene.
3. pCAG-hCas9: Expression vector for hCas9 (human codon optimized Cas9) under the CAG promoter (#51142, Addgene).
4. Genomic DNA extraction kit.
5. Thermal cycler.
6. PCR reagents.
7. PCR primers flanking the targeted region.
8. T7 Endonuclease I assay kit such as GeneArt Genomic Cleavage Detection Kit (Thermo, cat no A24372).

3 Methods

3.1 sgRNA Design

1. Obtain exon sequence from genome database such as UCSC Genome Browser.
2. Find highly specific 20 bp target sequences (*see* **Note 1**) on the locus that you intend to edit using a CRISPR/Cas9 target design tool such as CRISPRdirect (*see* **Note 2**). Where the 5′ end of the selected sgRNA sequence is not guanine, change to guanine (*see* **Note 3**). Selected sequence is defined as **S**, and the antisense complement of **S** is defined as **AS**.
3. Synthesize the following oligonucleotides with the sequences described below (*see* **Note 4**).

XgRNA-S: 5′-CACC**S**-3′

XgRNA-AS: 5′-AAAC**AS**-3′

3.2 Preparation of Cassette DNA

1. Make mix as follows.

10 × PCR buffer	1 μL
XgRNA-S (100 pmol/μL)	4.5 μL
XgRNA-AS (100 pmol/μL)	4.5 μL

2. Incubate at 95 °C for 5 min, 37 °C for 15 min, and r.t. for 15 min, for annealing of these oligonucleotides to produce the insert cassette DNA (Fig. 2).

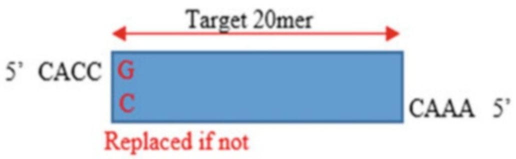

Fig. 2 Design of insert cassette DNA used for construction of gRNA vector. If the 5′ end of the target sequence is not G in the sense strand (C in antisense strand), these residues should be changed to G and C, respectively

3.3 Preparation of Vector

1. Digest the plasmid, gRNA cloning vector BbsI, with BbsI at 37 °C for 3 h.
2. Purify gRNA cloning vector BbsI following agarose gel electrophoresis with gel extraction kit (*see* **Note 5**).

3.4 Ligation

Ligate 1 μL of oligonucleotide mixture (prepared in Subheading 3.2) and 100 ng of gRNA-cloning vector BbsI digested with BbsI (prepared in Subheading 3.3) with T4 DNA Ligase at 16 °C for 4 h ~ O/N.

3.5 Transformation and Confirmation of the Clone

1. Transform the ligation mixture from Subheading 3.4 into *Escherichia coli* competent cells.
2. Culture on LB agar with kanamycin overnight at 37 °C.
3. Pick a few colonies and perform PCR amplification followed by DNA sequencing analysis to confirm the sequence. Primers used for colony PCR primers and sequence primers are Universal M13 Forward (−20) and Universal M13 Reverse. Primer used for sequencing is Universal M13 Forward (−20).

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTA
CCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCAT
ATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACAC
AAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGT
TTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTT
GAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC
gxxxxxxxxxxxxxxxxxxxGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTA
GTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGAC
CCAGCTTTCTTGTACAAAGTTGGCATTA

*Target is indicated in red letter.

4. Culture the selected colony in LB with kanamycin overnight at 37 °C.
5. Purify the plasmid with a plasmid DNA purification kit.

3.6 Validation of gRNA

1. Transfect the constructed gRNA vector into cells (*see* **Note 6**) with an equal amount (μg) of pCAG-hCas9 using a lipofection reagent.
2. Recover transfected cells 2 days after transfection.
3. Extract genomic DNA by using genomic a DNA extraction kit.
4. Perform PCR using primers flanking the targeted region.
5. Perform T7 Endonuclease I assay [5] using a kit (*see* **Note 7**).

4 Notes

1. Do not include the PAM sequence (NGG) in the target 20 bp sequence.
2. CRISPOR (http://crispor.tefor.net/) is also useful for the selection of the target sequences.
3. It is necessary for the first transcribed base to be guanine (G) because of the transcription initiation requirement of the human U6 promoter.
4. The sequence "CACC" and "AAAC" are for annealing to the protruding ends of the gRNA vector produced by cleavage with BbsI.
5. This step is for the removal of undigested circular plasmid.
6. We usually use mouse ES cells for mouse genes. In the case of other animals, use appropriate cells.
7. Restriction enzyme digestion assay can be also used for validation of gRNA if the target has a restriction enzyme recognition sequence around the Cas9 cleavage site located 3-nt upstream of the PAM sequence. In this assay, digest the PCR products with this restriction enzyme. If PCR product has insertion or deletion mutation, this restriction enzyme recognition sequence is disrupted. Analyze the PCR products by gel electrophoresis to determine mutation efficiency. The mutated DNA fragment cannot be cleaved by the restriction enzyme, while the non-mutated DNA fragment can be cleaved by the enzyme. Therefore, the mutation rate is calculated as $u/(c + u)$ where c = intensity of cleaved band and u = intensity of uncleaved band.

Acknowledgments

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Chapter 4

SNPD-CRISPR: Single Nucleotide Polymorphism-Distinguishable Repression or Enhancement of a Target Gene Expression by CRISPR System

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Abstract

A wide range of diseases, including cancer, autoimmune diseases, or neurodegenerative diseases, have been associated with single nucleotide mutations in their causative genes. Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system is a flexible and efficient genome engineering technology widely used for researches and therapeutic applications which offers immense opportunity to treat genetic diseases. The complex of Cas9 and the guide RNA acts as an RNA-guided endonuclease. Cas9 recognizes a sequence motif known as a protospacer adjacent motif (PAM), and then the guide RNA base pairs with its proximal target region of 20 nucleotides with sequence complementarity. Here we describe the procedure named single nucleotide polymorphism-distinguishable (SNPD)-CRISPR system which can suppress or enhance the expression of disease-causative gene with single nucleotide mutation distinguished from its wild-type. In this study, we used HRAS, one of most famous cancer-causative genes, as an example of a target gene.

Key words SNPD-CRISPR, dCas9, KRAB, VP64, PAM, Seed region, Single nucleotide mutation, HRAS

1 Introduction

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is an adaptive immune defense system in bacteria and archaea [1–3]. Cas9 is an RNA-guided endonuclease that cleaves double-strand DNA-bearing sequence complementary to a 20-nucleotide segment of the guide RNA (Fig. 1a). The presence of a short protospacer adjacent motif (PAM) is strictly required for Cas9 binding to recruit a synthetic single-guide RNA (sgRNA), mimicking the natural dual

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Shohei Maruyama and Takashi Kusakabe contributed equally with all other contributors.

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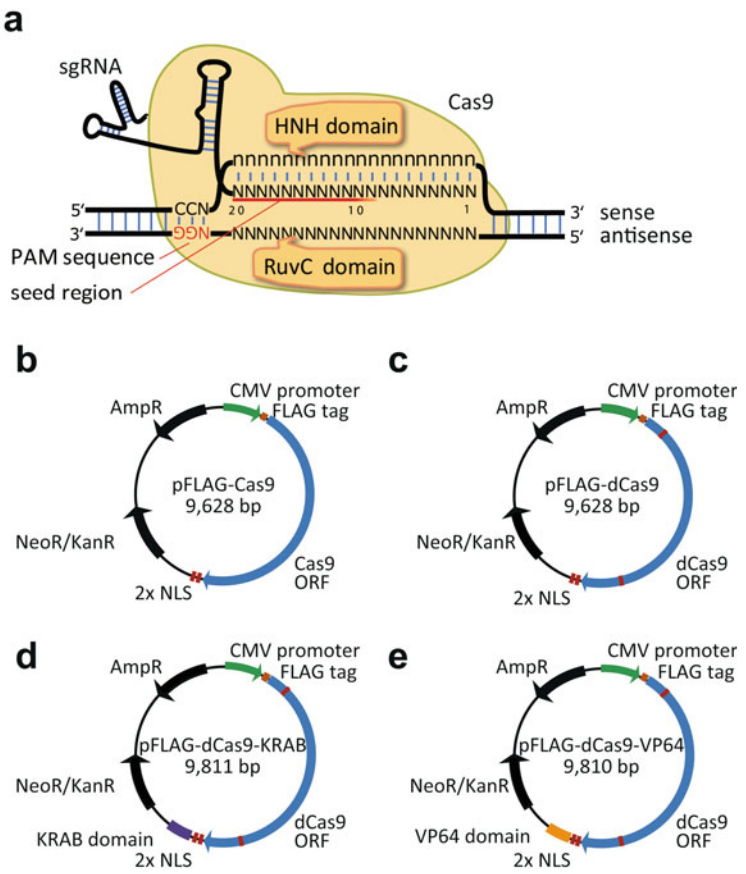

Fig. 1 Schematic presentation of CRISPR/Cas9 system and Cas9 expression constructs used in this study. (**a**) In CRISPR/Cas9 system, Cas9 protein recognizes PAM sequence, and sgRNA involved in Cas9 recognizes the adjacent target sequence of 20-nucleotide long. In these 20 nucleotides, 8–12 nucleotides adjacent to PAM sequence are considered to be particularly important for target recognition and called as seed region. The HNH and RuvC nuclease domains of Cas9 protein induce DSB which is repaired either by NHEJ or HDR. In dCas9 protein, both of HNH and RuvC domains are mutated, and dCas9 has no cleavage activity. The plasmids structures containing unified backbones: (**b**) pFLAG-Cas9, (**c**) pFLAG-dCas9, (**d**) pFLAG-dCas9-KRAB, and (**e**) pFLAG-dCas9-VP64

trans-activating CRISPR RNA (tracrRNA)-CRISPR RNA (crRNA) structure, to the proximal target region with sequence complementarity [4]. The subsequent base pairing between the guide RNA and target DNA and associated conformational changes induce double-strand break (DSB) at specific DNA site using HNH and RuvC nuclease domains of Cas9 protein [5–7]. The DSB is then repaired either by error-prone nonhomologous end joining (NHEJ), which creates small insertion deletions (indels), or by homology-directed repair (HDR), which can be used to generate precise genomic modifications if a homologous repair template is provided [8]. The CRISPR/Cas9 site-specific nuclease system has been exploited to develop potent tools for genome manipulation in various types of organisms. *Streptococcus pyogenes* Cas9 (SpCas9), which recognizes a canonical 5′-NGG-3′ PAM sequence [9], is the first Cas protein used for genome editing [7]. Due to its remarkable efficacy compared to other Cas proteins, it has become the most widely adopted tool in basic researches and potential clinical applications.

Single nucleotide mutations in functional genes are known to be the causes of many human diseases including cancers and hereditary disorders. Possible therapeutic approaches include repairing these mutations in vivo. It is well known that CRISPR/Cas9 is one of the most versatile systems, but it also exhibits a propensity of off-target activity [10–12]. CRISPR cytosine base editors [13], CRISPR adenine base editors [14], and CRISPR prime editors [15], and CRISPR base editors are also attractive procedures, because they efficiently introduce nucleotide alterations in the target DNA without the need for DSBs. However, the base types applicable for such approaches are limited, and their specificities remain a major challenge, because they are prone to off-target effect and transcriptome-wide editing. The alternative procedure to suppress the expression of mutated disease-causative genes may be the procedure using “dead” Cas9 (dCas9). dCas9 is developed to induce transcriptional repression by simply introducing the mutations into the HNH and RuvC catalytic domains of Cas9 to produce dCas9 with no nuclease activity [16]. dCas9 binds to the DNA target specifically recognized by the sgRNA without inducing DSB and interferes with transcriptional elongation, RNA polymerase binding, or transcription factor binding [17]. Thus, CRISPR/dCas9 is considered to have no significant off-target effects in terms of genomic DNA cleavage. Furthermore, dCas9 activity is improved by fusion to a variety of effector proteins that influence transcription or edit epigenetic marks. Krüppel-associated box (KRAB)-fused dCas9 (dCas9-KRAB) acts as a transcriptional repressor used to suppress the expression of target genes. KRAB acts with KRAB-associated protein 1 (KAP1) co-repressor, thereby recruits histone deacetylases (HDACs) and histone methyltransferases (HMTs) to promote heterochromatin formation [18–20] that results in the reduction of the chromatin accessibility in the promoter regions [21, 22]. In contrary, dCas9 fused with VP64 (dCas9-VP64) acts as a transcriptional activator. VP64 is tetramer of herpes simplex virion protein 16 (VP16), which is known to facilitate the steps required for open transcription complex formation [23] and increases the processivity of RNA polymerase II [24]. Thus, it may be possible to efficiently suppress or promote the expression of mutated genes by dCas9 in collaboration with effectors.

In this study, we utilized the mutated region of HRas proto-oncogene, GTPase (HRAS) as the target sequence. HRAS is one of the famous Ras family oncogenes, and mutation is observed in various cancers including 30% of squamous cell carcinoma. c.37G>C, in which nucleotide G at position 37 from the translation initiation site is changed to C, is the most popular causative mutation observed in about 17% of total HRAS mutations based on the Catalogue of Somatic Mutations in Cancer (COSMIC) [25]. We show that a single nucleotide polymorphism-distinguishable (SNPD) CRISPR system is a beneficial procedure to specifically suppress or enhance the disease-causing gene expression with single nucleotide mutation, distinguished from its wild-type.

2 Materials

2.1 Cas9 Expression Constructs (Fig. 1b–e)

1. pFLAG-Cas9, constructed from hCas9 (Addgene #41815) [26], in which the codon of SpCas9 is optimized for expression in human cells (hCas9) and FLAG tag is added.
2. pFLAG-dCas9, constructed from Cas9m2 (Addgene #47314) [26] whose active sites for cleavage are inactivated, but other backbone is completely same as that of hCas9.
3. pFLAG-dCas9-KRAB, constructed from pHR-SFFV-dCas9-BFP-KRAB (Addgene #46911) [27], in which dCas9 is fused with the KRAB domain having a transcriptional repressive function at the C-terminus.
4. pFLAG-dCas9-VP64, constructed from pMSCV-LTR-dCas9-VP64-BFP (Addgene #46912) [27], in which dCas9 is fused with VP64 domain having a transcription promoting function at the C-terminus.
5. PCR primers used for insertion of FLAG tag into hCas9 and Cas9m2 are 5′-TGACGACGATAAGATGGACAAGAAGTACTCCATTGGG-3′ and 5′-TCCTTGTAATCCATGGTGGCAAGGGTTCGATC-3′ (underline indicates FLAG tag region).
6. PCR primers used for amplification of the N-terminal nuclear localization signal (NLS) and VP64 region from pMSCV-LTR-dCas9-VP64-BFP are 5′-GTGTCTAGATCCGGATCTCCG-3′ (underline indicates *Xba* I site) and 5′-TTAGCTAGCACTACCTAGCATATCTAGATC-3′ (underline indicates *Nhe* I site).
7. PCR primers used for deletion of VP64 region are 5′-GCTAGCTGAAAGGGTTCGATCC-3′ and 5′-ACTACCAACTTTGCGTTTCTTTTTCG-3′.
8. PCR primers used for amplification of NLS and KRAS region from pHR-SFFV-dCas9-BFP-KRAB [27] are 5′-AGTTCTAGAGTGACCTTCAAGGATG-3′ (underline indicates *Xba* I

site) and 5′-TTAGCTAGCGGGCTCTTCTCC-3′ (underline indicates *Nhe* I site).

9. PCR primers used for amplification of NLS and VP64 region from pMSCV-LTR-dCas9-VP64-BFP [27] are 5′-GTGTCTAGATCCGGATCTCCG-3′ (underline indicates *Xba* I site) and 5′-TTAGCTAGCACTACCTAGCATATCTAGATC-3′ (underline indicates *Nhe* I site).

2.2 Generation of sgRNA Expression Construct (Fig. 2a)

1. pENTR.sgEmpty, constructed from pSuperCRISPR [28] and pENTR (Invitrogen).
2. Gel extraction kit such as Monarch DNA Gel Extraction Kit (New England Biolabs).
3. Oligonucleotides for generating sgRNA for HRAS_39–58 region are 5′-GATCCCGTCAGCGCACTCTTGCCCACA-3′ and 5′-AAACTGTGGGCAAGAGTGCGCTGACGG-3′ (underline indicates the complementary sequence of the overhang of *Bbs* I cleavage site, double underline indicates the sgRNA region for HRAS_39–58, and G in the square indicates the transcription start site of RNA polymerase III (Pol III)).
4. Annealing buffer (2×): 40 mM NaCl, 20 mM Tris–HCl, pH 7.5.
5. PCR kit such as AmpliTaq Gold PCR Master Mix 2× (Applied Biosystems).
6. PCR primers used for colony PCR are 5′-GTCGCTATGTGTTCTGGGAAATCA-3′ and 5′-CCAGGAAACAGCTATGACCATG-3′.
7. Sequencing kit such as BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).
8. ABI PRISM 3130xl genetic analyzer (Applied Biosystems).
9. Plasmid extraction kit such as Plasmid Midi Kit (QIAGEN).

2.3 Generation of Reporter Assay Constructs Expressing Firefly Luciferase (Fig. 2b)

1. pGL3-wild-type (WT) HRAS-5′, a target sequence of nucleotide position 36–58 of WT HRAS CDS (WT HRAS_36–58) was inserted into *Nco* I site at the 5′ side (two nucleotides upstream of translation initiation site) of firefly *luciferase* gene of pGL3-Control in the same direction of non-template, sense DNA. Oligonucleotides used for generating the target site are 5′-CATGCGGTGTGGGCAAGAGTGCGCTGA-3′ and 5′-CATGTCAGCGCACTCTTGCCCACACCG-3′ (underline indicates *Nco* I site).
2. pGL3-WT HRAS-3′, a target sequence of WT HRAS_36–58 is inserted into *Xba* I site at the 3′ side (two nucleotides downstream of translation termination site) of firefly *luciferase* gene in the same direction of sense DNA. DNA. Oligonucleotides

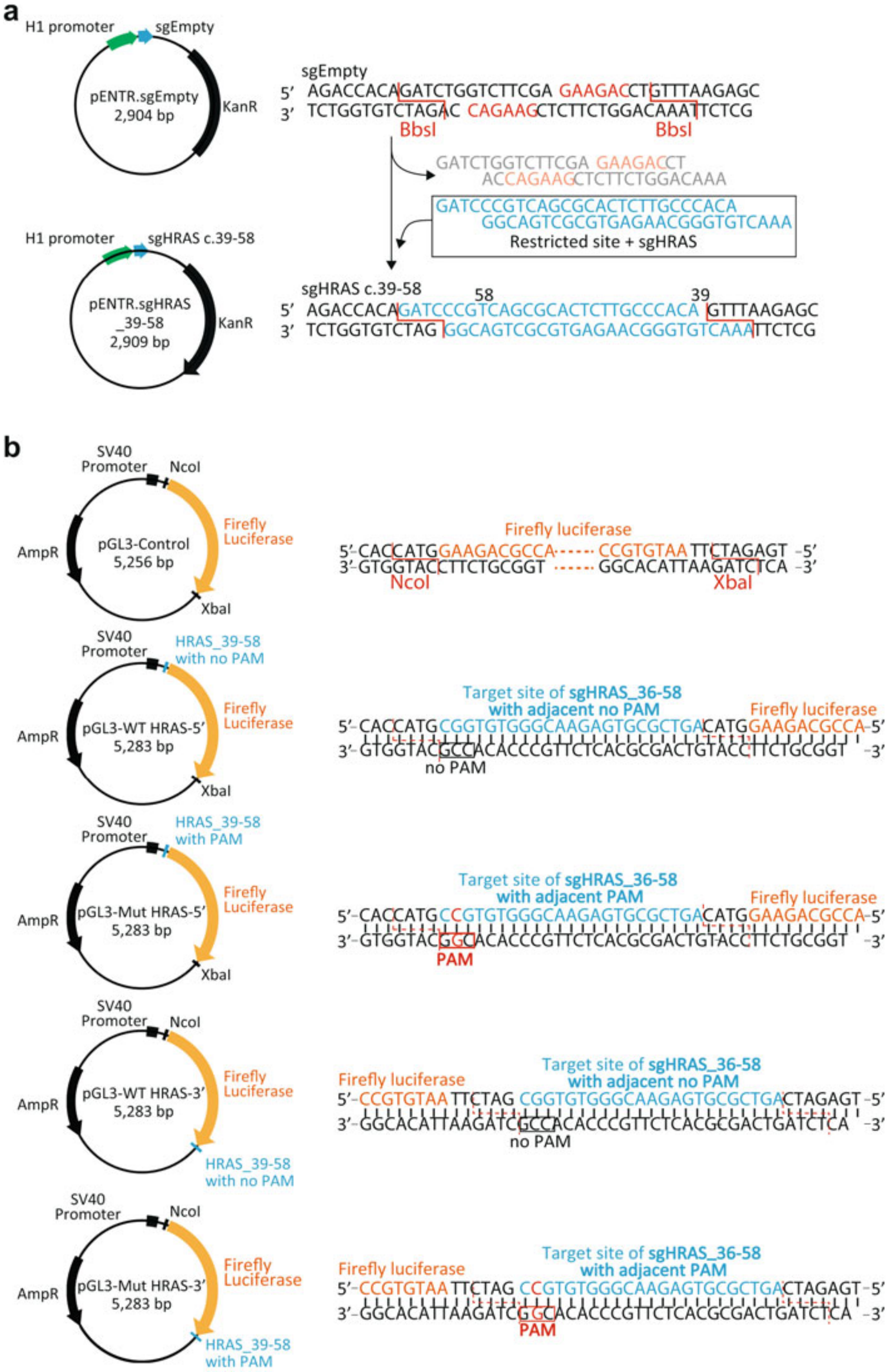
a
H1 promoter
sgEmpty
pENTR.sgEmpty
2,904 bp
KanR
sgEmpty
5′ AGACCACAGATCTGGTCTTCGA GAAGACCTGTTTAAGAGC
3′ TCTGGTGTCTAGAC CAGAAGCTCTTCTGGACAAATTCTCG
BbsI
BbsI
GATCTGGTCTTCGA GAAGACCT
ACCAGAAGCTCTTCTGGACAAA
GATCCCGTCAGCGCACTCTTGCCCACA
GGCAGTCGCGTGAGAACGGGTGTCAAA
Restricted site + sgHRAS
H1 promoter
sgHRAS c.39-58
pENTR.sgHRAS _39-58
2,909 bp
KanR
sgHRAS c.39-58
58
39
5′ AGACCACAGATCCCGTCAGCGCACTCTTGCCCACA GTTTAAGAGC
3′ TCTGGTGTCTAG GGCAGTCGCGTGAGAACGGGTGTCAAATTCTCG
b
SV40 Promoter
NcoI
AmpR
pGL3-Control
5,256 bp
Firefly Luciferase
XbaI
Firefly luciferase
5′-CACCATGGAAGACGCCA······CCGTGTAA TTCTAGAGT -5′
3′-GTGGTACCTTCTGCGGT ······ GGCACATTAAGATCTCA -3′
NcoI
XbaI
SV40 Promoter
HRAS_39-58 with no PAM
AmpR
pGL3-WT HRAS-5′
5,283 bp
Firefly Luciferase
XbaI
Target site of sgHRAS_36-58
with adjacent no PAM
Firefly luciferase
5′-CACCATGCGGTGTGGGCAAGAGTGCGCTGACATGGAAGACGCCA-5′
3′-GTGGTACGCCACACCCGTTCTCACGCGACTGTACCTTCTGCGGT -3′
no PAM
SV40 Promoter
HRAS_39-58 with PAM
AmpR
pGL3-Mut HRAS-5′
5,283 bp
Firefly Luciferase
XbaI
Target site of sgHRAS_36-58
with adjacent PAM
Firefly luciferase
5′-CACCATGCCGTGTGGGCAAGAGTGCGCTGACATGGAAGACGCCA-5′
3′-GTGGTACGGCACACCCGTTCTCACGCGACTGTACCTTCTGCGGT -3′
PAM
SV40 Promoter
NcoI
AmpR
pGL3-WT HRAS-3′
5,283 bp
Firefly Luciferase
HRAS_39-58 with no PAM
Firefly luciferase
Target site of sgHRAS_36-58
with adjacent no PAM
5′-CCGTGTAA TTCTAG CGGTGTGGGCAAGAGTGCGCTGACTAGAGT -5′
3′-GGCACATTAAGATCGCCACACCCGTTCTCACGCGACTGATCTCA -3′
no PAM
SV40 Promoter
NcoI
AmpR
pGL3-Mut HRAS-3′
5,283 bp
Firefly Luciferase
HRAS_39-58 with PAM
Firefly luciferase
Target site of sgHRAS_36-58
with adjacent PAM
5′-CCGTGTAA TTCTAG CCGTGTGGGCAAGAGTGCGCTGACTAGAGT -5′
3′-GGCACATTAAGATCGGCACACCCGTTCTCACGCGACTGATCTCA -3′
PAM

Fig. 2 Expression constructs of sgRNA and target fragment of HRAS. (**a**) Schematic structure of sgRNA expression construct. The upper pENTR.sgEmpty is an empty construct, and the lower pENTR.sgHRAS_39–58 is SNPD-sgRNA expression construct. pENTR.sgEmpty is digested by *Bbs* I and ligated with the oligonucleotide including sgHRAS_39–58. (**b**) Schematic structures of target reporter expression constructs: pGL3-WT HRAS-5′, pGL3-Mut HRAS-5′, pGL3-WT HRAS-3′, and pGL3-Mut HRAS-3′. pGL3-Control vector was digested with *Nco* I or *Xba* I, and HRAS_36–58 sequence without or with PAM was inserted into 5′ side or 3′ side of firefly *luciferase* gene

used for generating the target site are 5′-CTAGCGGTGTGGGCAAGAGTGCGCTGA-3′ and 5′-CTAGTCAGCGCACTCTTGCCCACACCG-3′ (underline indicates *Xba* I site).

3. pGL3-mutated (Mut) HRAS-5′, a target sequence of Mut HRAS_36–58 is inserted into the 5′ side of firefly *luciferase* gene in the same direction of sense DNA. Oligonucleotides used for generating the target site are 5′-CATGCCGTGTGGGCAAGAGTGCGCTGA-3′ and 5′-CATGTCAGCGCACTCTTGCCCACACGG-3′ (underline indicates *Nco* I site).
4. pGL3-Mut HRAS-3′, a target sequence of WT HRAS_36–58 is inserted in the 3′ side of firefly *luciferase* gene in the same direction of sense DNA. Oligonucleotides used for generating the target site are 5′-CTAGCCGTGTGGGCAAGAGTGCGCTGA-3′ and 5′-CTAGTCAGCGCACTCTTGCCCACACGG-3′ (underline indicates *Xba* I site).
5. PCR primers for confirmation of inserted sequences around *Nco* I site are 5′-CGATCTGCATCTCAATTAGTCAGC-3′ and 5′-CCGATAAATAACGCGCCCAACAC-3′, and those around *Xba* I site are 5′-GCTCCAACACCCCAACATC-3′ and 5′-CTGCATTCTAGTTGTGGTTTGTCC-3′.

2.4 Cell Culture, Transfection, and Luciferase Reporter Assay Reagents

1. HEK293 cells, derived from human fetal kidney cells.
2. Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) containing 1% penicillin/streptomycin.
3. Polyethyleneimine (PEI).
4. psiCHECK-1 vector (Promega).
5. Dual-Luciferase Reporter Assay System (Promega).
6. 1× passive lysis buffer (Promega).

3 Methods

3.1 Generation of Cas9 Expression Constructs

The backbones and the numbers of nuclear localization signals in the plasmids expressing SpCas9, dCas9, dCas9-KRAB, and dCas9-VP64 obtained from Addgene were unified (*see* **Note 1**, Fig. 1b–e).

3.1.1 pFLAG-Cas9 and pFLAG-dCas9 (Fig. 1b, c)

1. Insert FLAG tag into hCas9 and Cas9m2 plasmids, respectively, by inverted PCR using primers shown in **item 5** in Subheading 2.1. Transform *Escherichia coli* JM109 competent cells with each ligated plasmid, and incubate the cells on LB agar plate containing ampicillin overnight at 37 °C. Cultivate

the cells in LB broth containing 100 μg/mL ampicillin overnight at 37 °C, and purify the plasmid using Plasmid Midi Kit.

2. Insert an oligonucleotide containing *Nhe* I restriction enzyme site into the downstream of Cas9 ORF of hCas9 and dCas9 ORF of Cas9m2, respectively, by site directed mutagenesis using PCR primers, 5′-GCTAGCTGAAAGGGTTCGATCC-3′ (underline indicates *Nhe* I site) and 5′-CACCTTCCTCTTC TTCTTGGGGTCAGC-3′. Perform transformation and purification of the ligated plasmid using the same procedure as described in **step 1** in Subheading 3.1.1. Designate these plasmids as hCas9-Nhe I and Cas9m2-Nhe I, respectively.
3. Amplify the N-terminal nuclear localization signal (NLS) and VP64 region from pMSCV-LTR-dCas9-VP64-BFP by PCR using primers shown in **item 6** in Subheading 2.1.
4. Digest the amplified fragment containing NLS and VP64 with *Xba* I and *Nhe* I, and ligate with each of hCas9-Nhe I or Cas9m2-Nhe I fragment digested with only *Nhe* I (*see* **Note 2**). Perform transformation and purification of the ligated plasmid using the same procedure as described in **step 1** in Subheading 3.1.1.
5. From each plasmid, delete VP64 region by inversed PCR using primers shown in **item 7** in Subheading 2.1. Perform transformation and purification of the ligated plasmid using the same procedure as described in **step 1** in Subheading 3.1.1. Designate these plasmids as pFLAG-Cas9 and pFLAG-dCas9, respectively.

3.1.2 pFLAG-dCas9-KRAB (Fig. 1d)

1. Amplify the NLS and KRAB region from pHR-SFFV-dCas9-BFP-KRAB [27] by PCR using primers shown in **item 8** in Subheading 2.1.
2. Digest the amplified fragment from pHR-SFFV-dCas9-BFP-KRAB by Xba I and *Nhe* I, and ligate with Cas9m2-Nhe I fragment digested with only *Nhe* I and purify the ligated plasmid using the same procedure as described in **step 1** in Subheading 3.1.1. Designate this plasmid as pFLAG-dCas9-KRAB.

3.1.3 pFLAG-dCas9-VP64 (Fig. 1e)

1. Amplify the NLS and VP64 region from pMSCV-LTR-dCas9-VP64-BFP [27] by PCR using primers shown in **item 9** in Subheading 2.1.
2. Digest this amplified fragment with *Xba* I and *Nhe* I, and ligate with dCas9-Nhe I fragment digested with only *Nhe* I, and purify the ligated plasmid. Designate this plasmid as pFLAG-dCas9-VP64.

3.2 Generation of sgRNA Expression Construct

1. Digest pSuperCRISPR, derived from pSuper.retro.puro (Oligoengene) and pENTR [28], with the restriction enzymes, *EcoR* I and *Hind* III.
2. Ligate the fragment of pSuperCRISPR including sgRNA insertion region and the backbone of pENTR, and generate pENTR.sgEmpty (*see* **Note 3**). Transform JM109 competent cells with the ligated pENTR.sgEmpty, and incubate on LB agar plate containing 100 μg/mL kanamycin overnight at 37 °C. Cultivate the cells in LB broth containing kanamycin overnight at 37 °C, and purify the plasmid using Plasmid Midi Kit.
3. Digest pENTR.sgEmpty with the restriction enzyme *Bbs* I and subject to agarose gel electrophoresis, and purify the digested plasmid using the Monarch DNA Gel Extraction Kit.
4. Synthesize oligonucleotides containing HRAS_39–58 sequence and its complementary sequence having overhangs of *Bbs* I cleavage ends as shown in **item 3** in Subheading 2.2 (*see* **Note 4**). Anneal them in annealing buffer by lowering the temperature by 5 °C every 5 min from 95 to 25 °C.
5. Ligate the annealed oligonucleotides with *Bbs* I-digested pENTR.sgEmpty. Transform JM109 competent cells, and inoculate on LB agar plate with kanamycin overnight at 37 °C.
6. Perform colony PCR of the colonies proliferated on LB agar plate using AmpliTaq Gold PCR Master Mix 2× using primers shown in **item 6** in Subheading 2.2.
7. Subject a sequence reaction of a PCR product in which a band appeared at the desired position using the BigDye Terminator v3.1 Cycle Sequencing Kit, and analyze it using ABI PRISM 3130xl Genetic analyzer.
8. Perform transformation and purification of the plasmid having sgRNA for HRAS_39–58 using the same procedure as described in **step 1** in Subheading 3.1.1.
9. Designate the generated plasmid as pENTR.sgHRAS_39–58 (Fig. 2a). This encodes SNPD-sgRNA which can suppress the expression of Mut HRAS with single nucleotide mutation but not that of WT HRAS.

3.3 Generation of Reporter Assay Construct Expressing Target Sequence

1. Select HRAS_36–58 region including c.37G>C in the corresponding PAM region of Mut HRAS as target. WT HRAS_36–58 sequence is 5′-CGGTGTGGGCAAGAGTGC GCTGA-3′ (underline indicates not PAM sequence in the antisense strand of sgRNA target DNA strand), and Mut HRAS_36–58 sequence is 5′-CCGTGTGGGCA AGAGTGC GCTGA-3′ (underline indicates PAM sequence in the antisense DNA strand) (*see* **Notes 5** and **6**).

2. Synthesize each oligonucleotide of both strands of WT HRAS_36–58 and Mut HRAS_36–58 having either *Nco* I or *Xba* I restriction enzyme site as shown in **items 1–4** in Subheading 2.3, and anneal the both strands by the same procedure shown in **step 4** in Subheading 3.2 (*see* **Note 7**).
3. Digest pGL3-Control vector with *Nco* I or *Xba* I, and ligate with each of annealed WT HRAS_36–58 or Mut HRAS_36–58 double-stranded DNA oligonucleotide. Transform JM109 cells with the ligated plasmid, and inoculate on ampicillin-containing LB agar plate. Perform transformation and purification of the plasmid having sgRNA for HRAS_39–58 using the same procedure as described in **step 1** in Subheading 3.1.1.
4. At about 16 h after incubation at 37 °C, perform PCR for each colony using AmpliTaq Gold PCR Master Mix 2× using each PCR primer for *Nco* I or *Xba* I site shown in **item 5** in Subheading 2.3.
5. Separate each PCR product in agarose gel electrophoresis, and subject to sequence reaction of the band appeared at the desired position using BigDye Terminator v3.1 Cycle Sequencing Kit, and analyze by the ABI PRISM 3130xl Genetic analyzer.
6. Select the total for 4 types of reporter constructs. pGL3-WT HRAS-5′ and pGL3-WT HRAS-3′, in which a target sequence of WT HRAS_36–58 is inserted into either of 5′ side (two nucleotides upstream of translation initiation site) or 3′ side (two nucleotides downstream of translation termination site) of firefly *luciferase* gene of pGL3-Control in the same direction of sense DNA strand. pGL3-Mut HRAS-5′ and pGL3-Mut HRAS-3′, in which a target sequence of Mut HRAS_36–58 is inserted in either of 5′ side or 3′ side of firefly *luciferase* gene in the same direction of sense DNA strand (Fig. 2b).

3.4 Cell Culture and Transfection

1. Culture HEK293 cells in the DMEM containing 10% FBS containing 1% penicillin/streptomycin with 5% CO_2 at 37 °C.
2. Inoculate HEK293 cells on a well of 24-well plate at 1.0×10^5 cells/mL/well at about 16 h before transfection. Perform the transfection using PEI according to the manufacturer's procedure (*see* **Note 8**). For each well, transfect pGL3-Control containing each target sequence (500 ng), each of Cas9 expression construct (pFLAG-Cas9, pFLAG-dCas9, pFLAG-dCas9-KRAS, or pFLAG-dCas9-VP64), pENTR.sgHRAS_39–58, and psiCHECK-1 vector (10 ng) as an internal control.

3.5 Dual Luciferase Reporter Assay

1. Lyse the transfected cells with 1× passive lysis buffer 72 h after transfection (*see* **Note 9**), and measure the activities of *Renilla* luciferase and firefly luciferase using the Dual-Luciferase Reporter Assay System.

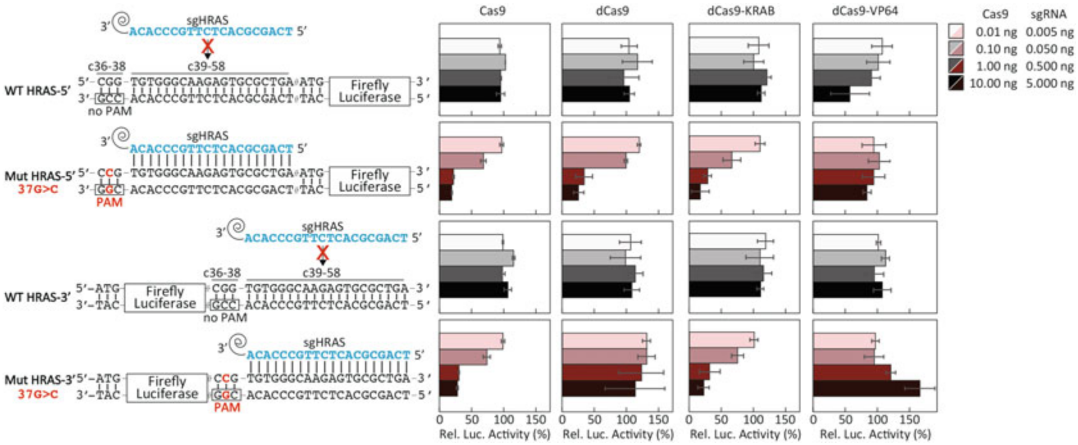

Fig. 3 Suppression activities of SNPD-sgHRAS on WT and Mut HRAS_36–58 by various types of Cas9 measured using Dual luciferase reporter assays. HEK293 cells were co-transfected with expression construct of Cas9 (pFLAG-Cas9), dCas9 (pFLAG-dCas9), dCas9-KRAB (pFLAG-dCas9-KRAB), or dCas9-VP64 (dCas9-VP64) (0.01, 0.1, 1.0, 10.0 ng/well), and 500 ng/well of each target expression construct of WT HRAS-5′ (pGL3-WT HRAS-5′), Mut HRAS-5′ (pGL3-Mut HRAS-5′), WT HRAS-3′ (pGL3-WT HRAS-3′), or Mut HRAS-3′ (pGL3-Mut HRAS-3′), and SNPD-sgHRAS expression construct (pENTR.sgHRAS_39–58) (0.005, 0.05, 0.5, 5.0 ng/well), along with 10 ng/well of an internal control psiCHECK-1 vector. Cells were harvested 72 h after transfection, and relative luciferase activities were measured. The data were averaged using three independent experiments

2. Calculate the relative luciferase activity by dividing the activity of firefly luciferase encoded from the target-expression construct by that of control *Renilla* luciferase.
3. Normalize the relative luciferase activity of each sample by the relative luciferase activity determined using pENTR.sgEmpty, which is a control vector that does not express sgRNA (Fig. 3) (*see* **Note 10**).

4 Notes

1. All of the pFLAG-Cas9, pFLAG-dCas9, pFLAG-dCas9-KRAB, and pFLAG-dCas9-VP64 expression constructs are expected to function in the nucleus. One or two NLS are essential to translocate into the nucleus of these proteins. In this study, two NLS domains were inserted into all of the expression plasmids of these proteins.
2. Restriction enzyme recognizes a specific sequence of DNA. *Nhe* I and *Xba* I recognize 5′-GCTAGC-3′ and 5′-TCTAGA-3′, respectively, and produce a double-stranded cleavage with cohesive (sticky) ends. The cohesive ends of both restriction enzymes are same “CTAG.” Therefore, if *Nhe* I cut fragment is ligated with *Xba* I cut fragment, both restriction enzyme sites

are collapsed. In this case, the amplified fragment containing NLS and VP64 from pMSCV-LTR-dCas9-VP64-BFP was cut with *Xba* I and *Nhe* I and ligated with each of hCas9-Nhe I or Cas9m2-Nhe I fragment digested with only *Nhe* I. Therefore, *Xba* I sites become no more functional by ligation reaction.

3. A widely used sgRNA expression plasmid has a Pol III-driven promoter such as U6 or H1 promoter, because the length of sgRNA is as small as about 100 nucleotides long. The pENTR. sgHRAS has H1 pol III promoter, and the length of transcribed sgRNA is 98 nucleotides.
4. For transcription start by pol III, it is preferable to put G residue at the start position.
5. In this study, we used the target sequence in which the single nucleotide mutation is located in the PAM sequence. However, it may be also possible to set the position of single nucleotide mutation in the guide RNA seed region (about 10-bp PAM--proximal region), since it has been revealed that the seed region strictly determines target specificity via sequence complementarity [7, 29].
6. The target sequence directed by CRISPR system is selectable using CRISPRdirect [30]. CRISPRdirect is a simple and functional web server for selecting rational CRISPR/Cas targets from an input sequence. Appropriate target sequence can be obtained by putting the mutated gene sequence into CRISPRdirect. The mutated sequence could be chosen from the genic region as well as its promoter or other transcription starting site (TSS) proximal regions.
7. pGL3-Control (Promega), expressing firefly luciferase, was used as reporter plasmid containing each of target sequence. In this study, two restriction enzyme sites were used for insertion sites of target sequences: *Nco* I site at the two nucleotides upstream of firefly *luciferase* translation initiation site and *Xba* I site at the two nucleotides downstream of firefly *luciferase* translation termination site. Any other sites are also useful.
8. In this study, we used PEI as transfection reagent. However, the other transfection regents are useful for transfection of pENTR.sgHRAS, such as Lipofectamine2000 (Invitrogen).
9. If many cell debris are observed in the transfected cells, it is preferable to remove such debris clearly before the lysis of the cells by 1× passive lysis buffer to measure the luciferase activity correctly.
10. Cas9 suppressed the expression of firefly *luciferase* gene when HRAS_39–58 with PAM is existing at both upstream and downstream of firefly *luciferase* ORF. dCas9 suppressed the expression of firefly *luciferase* gene when HRAS_39–58 with

PAM is existing at upstream of firefly *luciferase* ORF, but its effect is no or weak at downstream. The suppression effects of dCas9 is enhanced by dCas9-KRAB. The enhancement of firefly luciferase expression is induced by dCas9-VP64 when HRAS_39–58 with PAM is existing at downstream but not at upstream, suggesting that dCas9 involved in dCas9-VP64 may suppress the expression of firefly *luciferase* gene when HRAS_39–58 with PAM is existing at upstream but such effect may be weak at downstream.

Acknowledgement

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Chapter 5

Utilizing Large Functional and Population Genomics Resources for CRISPR/Cas Perturbation Experiment Design

Qingbo S. Wang and Kumiko Ui-Tei

Abstract

Genome sequencing technologies have rapidly evolved in the past decades, enabling us to interpret the human genome through multiple perspectives, ranging from cross-species comparisons, naturally occurring variation in health and disease state to regulatory mechanisms.

Although such perspectives are all informative to narrow down the list of genes or variants for perturbation experiments based on specific biological aims, utilizing multiple sources of information is often challenging in practice.

In this chapter, we provide an overview of major large-scale functional and population genomics resources, followed by a practical example of selecting target variants for genetic perturbation experiments involving genome engineering techniques such as CRISPR/Cas.

Key words GWAS, Fine-mapping, Variant annotation, Target selection

1 Introduction

Large-scale functional and population genomics studies have highly improved our ability to interpret a specific variant in the human genome. For example, genome-wide association studies (GWAS) [1] highlight a set of genomic regions containing multiple variants associated with various phenotypes, and overlaying information from epigenetic resources such as DNA methylation and histone modifications [2, 3] further elucidates relevant biological processes, connecting genomic variants and phenotypic observations through the lens of molecular biology (Figs. 1 and 2).

These facts indicate that such information can in turn be utilized to nominate a set of variants that are most likely to be relevant to the biological processes one is interested in.

Extracting relevant information from large databases often requires nontrivial efforts, such as parsing a large data frame and performing complicated statistical analyses, while such efforts could

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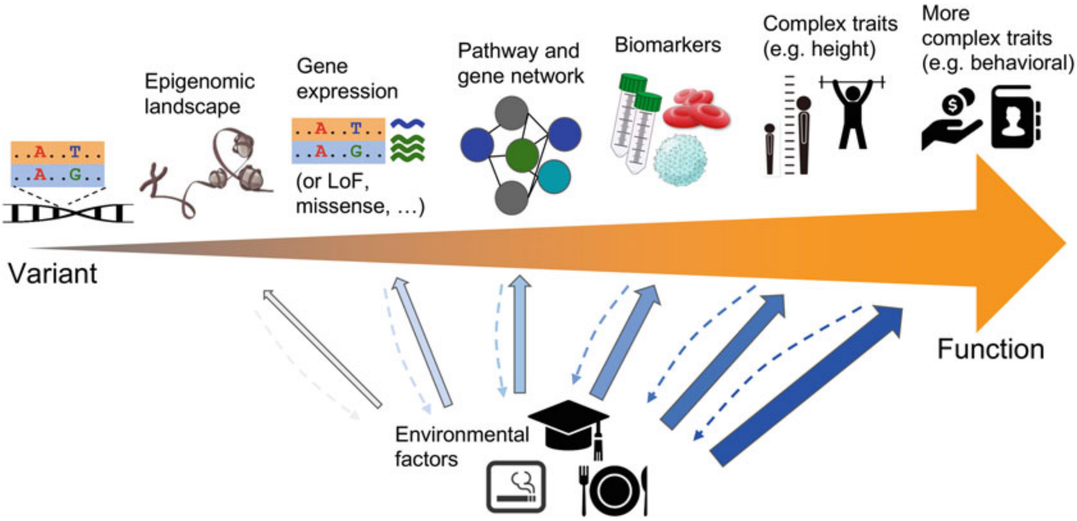

Fig. 1 A model of a variant to complex functions. A genetic variant could contribute to complex phenotypes through regulation of genes at various levels, which are often accompanied by modification of epigenetic landscapes such as histones. Environmental factors interact with human traits at different levels ranging from molecular to extremely complex phenotypes, often limiting the genetic heritability one can observe through association studies

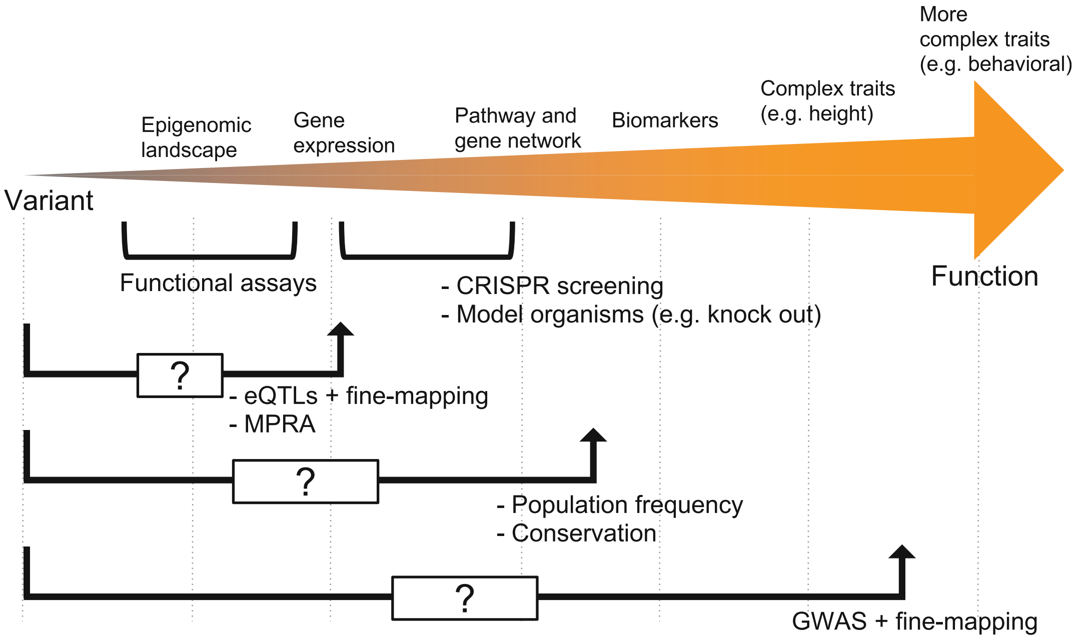

Fig. 2 The scope where different datasets cover in the spectrum of variant to function. Functional assays are utilized to uncover the function of a variant in the context of cellular and molecular biology, although they do not directly highlight the phenotypic consequences in humans. Observation of naturally occurring variations such as in GWAS combined with statistical fine-mapping, on the other hand, leaves the biology between a genetic variant to complex phenotypes as a black box. Combining pieces of evidence from multiple observations are thus thought to be important

be minimized by utilizing user-friendly browsers or post-cleaned summary data. Targeting researchers with various levels of expertise in computational biology, this manuscript intends to demonstrate ways of utilizing large databases while minimizing complicated data processing steps.

Specifically, in the Subheading 2, we classify existing genomics resources into different categories, with highlights of one or more specific databases that are readily available for researchers (without advanced knowledge of bioinformatics or large-scale computational genomics) for each category. Then in the Subheading 3, we will walk through the process of selecting target variants for a genetic perturbation experiment design, with an example of designing a CRISPR/Cas genome perturbation experiment for investigation of blood-related phenotypes. Further practical advice will be covered in the Subheading 4.

2 Materials

We classified major genomics resources for variant interpretation into two categories, each divided into four subcategories (*see* **Note 1**), as listed below and in Table 1. The first category is based on observations of genome variation in human and other species, while the second category is based in lab experiments (Fig. 3).

Table 1
Overview of available resources divided into subcategories

Category	Subcategory	Examples	Pros	Cons
Natural variation	Population frequencies	gnomAD Clinvar	Large effect sizes	Non-deep phenotype
	Complex trait GWAS	GWAS catalog Open Targets	Diverse phenotypes	Limited effect sizes
	eQTLs	GTEx eQTL catalogue	Tissue-specific interpretation	Not always trait-associated
	Comparative genomics	PhyloP primAD	Evolutionary contexts	No detailed phenotypes
Lab experiments	Functional assays	ENCODE ROADMAP	Genome-wide	Intermediate phenotypes
	MPRA	MPRA portal	Single variant resolution	Not in the native genome context
	Perturbations	TKO Library ABC	Native genome editing (in vitro)	Not always single variant resolution
	Model organisms	IMPC MGI	In vivo	Not human Low throughput

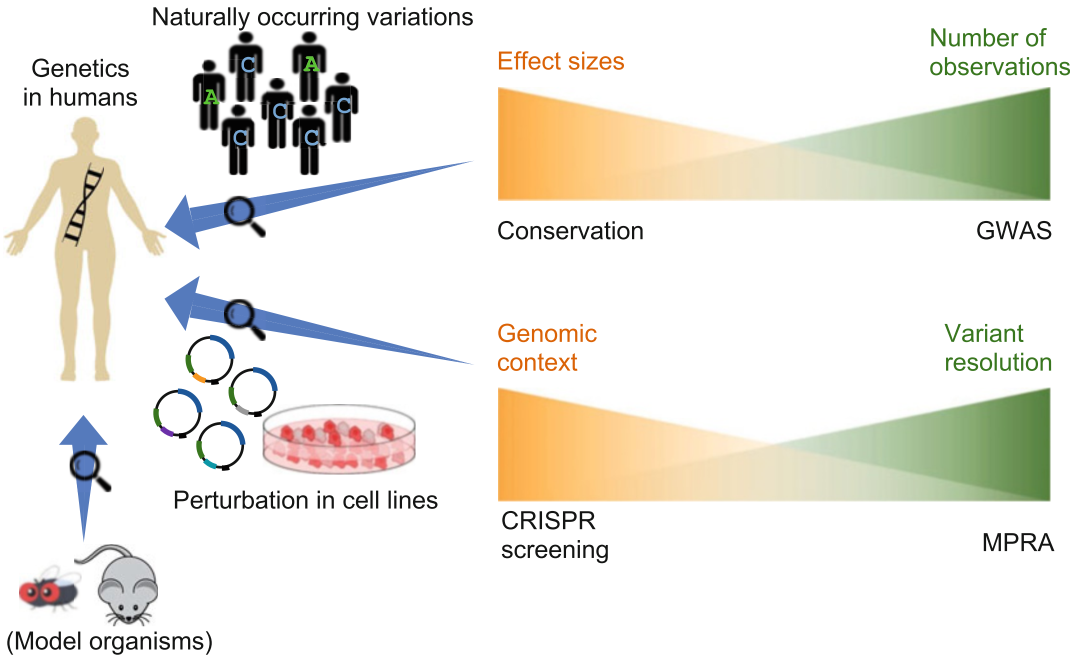

Fig. 3 Trade-offs between different high-throughput methods for investigating human genetics. As proxies of direct perturbation of the genome in a human body, researchers typically perform (1) observational studies on naturally occurring variations (top), or (2) perturbation in cell cultures (middle, or model organisms; bottom). For (1), GWAS is more suited for understanding relatively small effects of common genetic variations on diverse phenotypes, while conservation or other rare-variant studies are more suited for nominating variants with large effect sizes. For (2), MPRA allows perturbation at single variant resolution in a plasmid, while typical pooled CRISPR screening allows perturbation in the native genome, although at gene-level rather than at a single variant resolution

2.1 Natural Variation in Human Population and Other Species

2.1.1 Population Frequencies in Health and Disease

Millions of variants exist in our human genome; some are commonly seen in the population, while others are extremely rare (e.g., only one in a million people on average carries the variant). Variants leading to severe health defects are unlikely to be observed in a reference genome database with high frequency, and are more likely to be annotated in a disease-focused database. gnomAD (https://gnomad.broadinstitute.org) [4] is the most commonly used population reference database to date. Entering the variant ID in the browser, one can inspect the frequency of the variant in each population. Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/) [5] is a common database where researchers report variants observed in various disease patients. The browser allows flexible search of variants by gene name, phenotype descriptions, or other various conditions, for variants with different levels of pathogenicity confidence. Combining gnomAD and Clinvar allows us to highlight potentially disease-causing variants as well as likely benign variants that serve as negative controls (*see* **Note 2**).

2.1.2 Association with Various Complex Human Traits (GWAS and Fine-Mapping)

Millions of genetic variants associated with thousands of phenotypes are available in different web portals such as Open Targets Genetics (https://genetics.opentargets.org) [6], Biobank Japan pheweb (https://pheweb.jp) [7], UK Biobank Genebass (https://genebass.org/) [8], and the GWAScatalog (https://www.ebi.ac.uk/gwas/) [1] curating more than 5000 studies (as of 2022/03). Open Target Genetics also displays information about multiple regulatory evidences of different variants, such as expression quantitative trait loci (eQTLs) that will be described next.

One caveat (*see* **Note 3**) is that a variant being associated with a phenotype does not necessarily mean that the variant is causal; in most cases, they are just in linkage disequilibrium (LD) with the true causal variant(s). We thus highly recommend overlaying information from fine-mapping study. Fine-mapping [9] is an attempt to quantify the probability of a variant (or set of variants) being causal to the phenotype, rather than just the association. In other words, fine-mapping quantifies the posterior inclusion probability (PIP) rather than just the association p-value. We presented fine-mapping of large biobank data [10] or eQTLs [11], and the results are available at (https://www.finucanelab.org/data).

2.1.3 Association with Gene Expression (eQTLs)

When testing the association between a genotype and a phenotype as in GWAS, the phenotype does not need to be the ones we "see" in our eyes; eQTLs are loci with genetic variations that are associated with gene (mRNA) expression, and is thought to be helpful in understanding the mechanisms where a variant affects a phenotype, especially in non-coding regions. GTEx (https://gtexportal.org/) [12] is one of the most common eQTL databases with a browser, displaying eQTLs for >50 tissues (*see* **Notes 4–6**). eQTL-catalog (https://www.ebi.ac.uk/eqtl/) [13] is another common one uniformly processing data from different study groups. Fine-mapping results of eQTLs in different populations are also available from previous works such as [11, 12, 14].

2.1.4 Natural Variation Across Species (Comparative Genomics)

So far we have reviewed insights from within-human natural variations. Expanding our scope, comparing the human (reference) genome with >100 different species allows us to understand how a variant has been important throughout evolutionary history. Genomic Evolutionary Rate Profiling (GERP), phyloP and fastCons are examples of estimation of evolutionary constraint through comparative genomics [15] (visible in, e.g., UCSC genome browser (*see* **Note 7**)). Inspired by gnomAD, primAD (primate genome aggregation database (https://primad.basespace.illumina.com)) is a browser allowing for visual comparison between the genome variation in humans and other primates. Such information would help researchers predict the effect of a variant on fitness.

2.2 Functional Genomics and Perturbation Experiments

2.2.1 Large-Scale Functional Genomics Assay

Histone mark landscape, chromatin accessibility, transcription factor binding, three-dimensional genome structure, and DNA methylation are all of high importance in evaluating a genomic variant in terms of biological processes, especially for non-coding variants. For example, a variant that disrupts a transcription factor binding motif is more likely to have an effect on a phenotype.

ENCODE [2] and ROADMAP [3] are the most commonly used large databases for epigenetic annotation of the human genome. JASPAR [16] is one of the many common TF-binding motif databases. These data are often combined with GWAS evidence to increase the confidence of fine-mapping [11] or to biologically interpret the collective effects of GWAS hits (e.g., Bulik-Sullivan et al. [17] allow us to prioritize the relevant tissue for GWAS results).

2.2.2 High-Throughput Reporter Assay

Phenotype-causal variants are often characterized by alterations of nearby gene expressions (i.e., *cis*-regulatory effects). Massively parallel reporter assay (MPRA) is a method that one can quantify the *cis*-regulatory effects of tens of thousands of variants in a high-throughput manner. Steps of MPRA involves designing thousands (or more) of synthetic ~200 bp sequences, insertion into a vector (reporter), transfection to cultured cells, and the comparison of the reporter gene expression with and without a variant. Although this is done with a relatively low proximity to the native human physiology (e.g., genomic contexts outside of the ~200 bp sequence oligos are not captured, and the oligos are not integrated to the genome; Fig. 3), data have shown that MPRA-positive variants are highly enriched for disease and other phenotype relevant variants. Summary of MPRA results are available in, e.g., Supplementary Data shown by Tewhey et al. [18]. Kircher et al. [19] provides a user-friendly portal, although only for a limited number of regions. We anticipate a genome wide-scale MPRA results portal will soon be available.

2.2.3 Human Cell Perturbations

Although not in the scale of MPRA, results from perturbation of the native genome rather than the plasmid utilizing CRISPR/Cas exist, such as [20] (saturation mutagenesis on *BRCA1* gene). Lowering the resolution from single variant to single gene level, genome-wide CRISPR/Cas screening has been commonly applied to test the essentiality of human (or mouse) protein coding genes in different conditions (e.g., [21]). Such screening techniques are extended utilizing CRISPR interference (CRISPRi) (e.g., Activity-by-Contact (ABC) [22]), allowing us to prioritize enhancer regions based on activity in the native genome context.

2.2.4 Knockout in Mouse and Other Model Organisms

Results of gene knockout experiments in mouse or other model organisms have been curated by the community (e.g., MGI [23] and IMPC [24]). These datasets combined with high-throughput essentiality screening results based on perturbation experiments in human cells, although most cases are limited in terms of resolution (i.e., not for variant interpretation), provide highly useful information at gene level.

3 Methods

Here we walk through a case of choosing a set of variants with likely blood-related phenotypic effects. We assume this situation would be applicable, for example, when a researcher is designing CRISPR/Cas perturbation experiments in human hematopoietic stem cells (Figs. 3 and 4).

3.1 Choosing Negative Controls

One can select a set of likely non-causal variants based on population frequency as well as other functional and GWAS information. Simplest "random" negative controls would be a random set of variants commonly observed in gnomAD (e.g., Minor Allele Frequency = MAF > 0.1 in all populations). Another set of negative

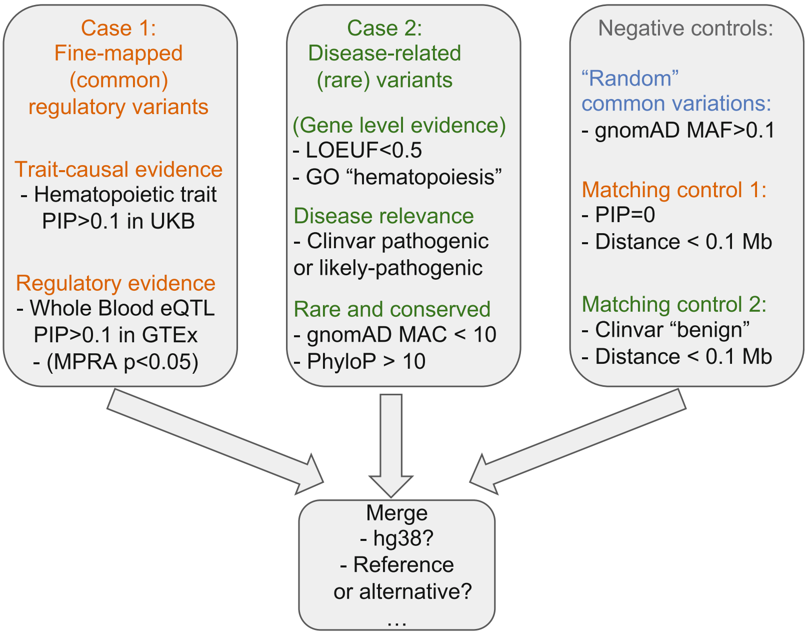

Fig. 4 An example of a workflow of selecting a set of variants for perturbation experiments. Target variants with functional evidence can be selected based on complex trait and eQTL fine-mapping studies (Case 1), or variant annotations in health and diseased state (Case 2). Corresponding negative controls are selected based on minor allele frequency (MAF), posterior inclusion probability (PIP, a proxy of the probability that a variant is causal to a phenotype), or Clinvar annotation status (MAC = minor allele count, and GO = Gene Ontology)

controls would be a set of variants that are associated with blood related traits but are thought to be non-causal; such variation could be obtained by filtering to PIP = 0 (or nearly equals 0) in fine-mapping studies and allows us to distinguish a functional and a non-functional variant both in a locus that are associated with the trait. Further control for specific epigenetic factors could be useful. For example, one can filter to variants in a DNAse peak but PIP = 0 to control for chromatin accessibility effects.

3.2 Gene Prioritization

Before considering single-variant resolution, one can narrow down the list of genes to those relevant to blood-related traits. For example, one can query by Gene Ontology (GO) term with key words "blood" or "hematopoiesis" to get a list of relevant genes. We assume this step is optional, as one might be interested in discovery of novel variants and genes associated with the phenotype.

3.3 Choosing Likely Functional Variants

Obtaining a list of variants associated with various blood-traits from GWAScatalog could be a first step (optional). From there, we can further narrow down the list of variants into smaller sets of candidates by (1) Filtering to PIP > threshold (e.g., 0.1) for "trait-causal" evidence, and (2) Intersect with eQTLs in whole-blood tissue (ideally with PIP > threshold), for "regulatory" evidence. Further evidence from lab experiments such as MPRA [18, 19] would be ideal.

3.4 Choosing Additional Exploratory Variants

Although Subheading 3.3. allows one to find a set of variants with multiple layers of evidence, one important note is that evidence from GWAS (and eQTL) studies are restricted to common variants, often with limited effect sizes that could be below the detection threshold in lab experiments (*see* **Note 3**). Thus, in addition, one may select variants with potentially large effects by (1) Selecting a list of non-benign variants in Clinvar, (2) Filtering to constrained genes (if a variant is coding) and rare variation in gnomAD as in Subheading 3.2, and (3) Filtering to conserved elements by thresholding with various conservation scores. These variants, although with thinner layers of population and functional evidence, could be the ones with largest effects.

3.5 Constructing a Library

Once the list of variants are finalized, one can put it into a single data frame (or simply an excel file), with each row corresponding to a variant (with an identifier in a specific genome build (*see* **Note 8**)) and columns for various annotations we have explored so far. The library based on the pipeline example (Fig. 4) is available online at (https://github.com/QingboWang/variant_design_wang_and_tei/) (*see* **Notes 9** and **10**). Once done, one should be ready for the next steps such as designing gRNA library (*see* **Note 11**).

Summary

In this chapter, we have reviewed major large-scale functional and population genomics resources with a practical example of combining information from such resources to narrow down a set of variants to study. Along with the increasing scale and quality of experiments and computational analysis, we anticipate the importance of integrating biological evidence from different layers continues to grow.

4 Notes

1. Although we classified types of genomics resources into a small number of classes in this manuscript, in reality those are non-independent. For example, functionally informed fine-mapping is a method that combines functional genomics annotations for fine-mapping of GWAS or eQTL signals [11]. In addition, we note that there exists a much wider variety of resources that are not covered in this manuscript.
2. Variant effect predictor (VEP) are commonly used to annotate variants with gnomAD frequency and additional features.
3. As an additional caveat, even when a variant of interest is a GWAS "hit," we need to be cautious of its implication by inspecting the effect size and direction. GWAS with large sample sizes are suited for finding variants with extremely small effect sizes (e.g., odds ratio <1.01), but replicating such small effects in experiments could be challenging.
4. When designing experiments, biological contexts such as the cell type are of prominent importance. Presenting a specific phenotype of interest in the population does not necessarily mean that the relevant phenotypes are observed in the cell type one is studying.
5. **Note 4** also reminds us of the fact that most functional genomics or eQTL resources are ascertained in a small number of specific cell types or tissues. Choosing functional genomics evidence that are as proximal to the biological context would be of high importance.
6. Resolution is another layer to consider. For example, thanks to the advance of single-cell RNA-seq resources, there is an increasing amount of evidence that regulatory effects can be highly specific to a certain cell type, even within a tissue.
7. UCSC genome browser (http://genome.ucsc.edu) is a great resource to integrate a large majority of the information described above into a single browser window. It also allows us to download the data in a table format.

8. Different databases use different identifiers for individual variants. For example, the human genome reference build a database is based on could be different: some databases are based on hg19 (e.g., Pheweb) or even older genome builds, while others are based on hg38 (e.g., GTEx/Open Target Genetics). Also, reference single nucleotide polymorphism identifier (rsID) is commonly used as an identifier for common variants, but rsIDs do not necessarily work as unique identifiers, and we thus discourage the usage of rsIDs when annotating variants, although this is common in practice.
9. When considering multiple proximal variants, taking into account the combination of those variants in terms of paternal or maternal alleles (i.e., haplotypes) [25] would be ideal. This is especially of high importance in the coding regions, where haplotype can drastically change the consequences of genetic variations [25].
10. Additional filtering should be applied based on the experimental design. For example, if the designed experiment is focused on protein coding regions, non-coding variants should be removed.
11. Editing the genome at near-single nucleotide resolution is not only extremely technologically difficult—additional challenges reside in minimizing off-target effects [26].

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Chapter 6

Generation of Genome-Edited Mice by Cytoplasmic Injection of CRISPR-Cas9 RNA

Takuro Horii and Izuho Hatada

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) gene editing enables rapid production of genome-edited animals. The Cas9/guide RNA (gRNA) component can be introduced into zygotes in several ways. Here, we provide an instructional guide for the generation of knockout mice using cytoplasmic injection of in vitro transcribed Cas9 RNA and gRNA.

Key words CRISPR-Cas9, Cytoplasmic injection, In vitro transcription, Microinjection, Mouse

1 Introduction

Novel genome-editing technologies using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins 9 (Cas9) (CRISPR-Cas9) system have emerged as effective tools for generating gene knockout and knock-in animals. The use of CRISPR-Cas9 has enabled rapid production of genome-edited animals including mouse, rat, rabbit, pig, monkey, and other species. In general, the system entails introduction of two components, Cas9 and a guide RNA (gRNA), into zygotes. Currently, Cas9/gRNA can be injected in multiple forms (DNA, RNA, or protein) into various locations within the cell (pronucleus or cytoplasm). The most convenient and direct method is pronuclear injection of plasmid DNA encoding Cas9 and gRNA. Expression vectors can be easily produced from large-scale culture of gene-modified bacteria, and many laboratories have established routine systems for the generation of transgenic mice that can be applied to the CRISPR-Cas9 system without any modifications. However, pronuclear injection of plasmid DNA has the risk of vector integration into the chromosome, albeit at a very low rate (4.3%) [1]. Furthermore, pronuclear injection causes physical damage to the

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nucleus, decreasing the rate of development both in vitro and in vivo [2]. Therefore, injection into the cytoplasm is an attractive alternative.

We previously showed that the cytoplasmic injection of RNA is the most efficient method for obtaining large numbers of normal blastocyst stage embryos, full-term pups, and mutant mice [2]. However, it should be noted that RNA is susceptible to degradation by contaminating RNase. Thus, microinjection must be performed carefully under RNase-free conditions. Moreover, in vitro transcription of RNA may be difficult and time-consuming for researchers who are not familiar with handling RNA. Fortunately, due to the widespread use of the CRISPR-Cas9 system, in vitro transcribed Cas9 RNA and gRNA are now commercially available. The prices of these reagents are getting cheaper every year, and the components are delivered to the user about a week after they are ordered. Thus, RNA injection can be performed as conveniently as DNA injection.

The present chapter serves as an instructional guide for cytoplasmic injection of CRISPR-Cas9 RNA into fertilized mouse eggs. Although in vitro transcribed Cas9 RNA and gRNA can be ordered from various manufacturers, we have nonetheless provided protocols for the in vitro transcription of these RNAs in your laboratory.

2 Materials

2.1 In Vitro Transcription

1. gRNA cloning vector (#41824, Addgene, Cambridge, MA, USA) [3].
2. pCAG-hCas9 (#51142, Addgene) [4].
3. High-fidelity DNA polymerase such as Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA).
4. PCR purification kit such as QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).
5. In vitro transcription kit for gRNA: MEGAshortscript T7 Kit (Life Technologies, Waltham, MA, USA).
6. In vitro transcription kit for Cas9: mMESSAGE mMACHINER T7 ULTRA Kit (Life Technologies).
7. RNA purification kit: MEGAclear Kit (Life Technologies).
8. Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) or another instrument for RNA quantification.

2.2 Embryo Collection and Culture

1. Mice, e.g., 4-week-old (female) and >8-week-old (male) C57BL/6 (B6) mice and 8–10-week-old (female) and >8-week-old (male) B6D2F1 mice.

2. Pregnant mare's serum gonadotropin (PMSG).
3. Human chorionic gonadotropin (hCG).
4. M16 embryo culture medium [5]: Add disodium EDTA (final concentration, 100 μM) before use.
5. M2 embryo culture medium [5].
6. M2-hyaluronidase medium: M2 medium containing hyaluronidase (300 U/mL).
7. Paraffin oil.
8. 35 and 60 mm plastic dishes.

2.3 Cytoplasmic Microinjection

1. Micropipet Puller for fabricating micropipets such as P-97/IVF (Sutter Instrument, Novato, CA, USA).
2. Microelectrode beveler for precision beveling of micropipet tips such as BV-10 (Sutter Instrument).
3. A microforge for bending the injection pipet and a fire-polishing holding pipet such as MF-900 (Narishige, Tokyo, Japan).
4. Glass micropipet such as Borosilicate Glass B100-75-10-PT (Sutter Instruments).
5. 100 mm cell culture dish.
6. Micromanipulation system.
7. Inverted microscope.

2.4 Embryo Transfer

1. Pseudopregnant foster mice such as ICR mice, >8 weeks of age.
2. Vasectomized mice such as ICR mice, >8 weeks of age.

2.5 Assay for Genome Modification

1. TA-cloning vector such as that supplied with the TOPO TA Cloning Kit (Thermo Fisher Scientific).

3 Methods

3.1 In Vitro Transcription

The in vitro transcription protocols for Cas9 mRNA and gRNA are described below, but it is more convenient and economical to purchase these reagents from a manufacturer of these reagents (*see* **Note 1**).

3.1.1 In Vitro Transcription of gRNA

1. We use gRNA cloning vectors as templates for in vitro transcription. Prepare the primer set shown below for in vitro transcription of gRNA. Insert 20 bp of the target sequence (the regions marked in bold) into the forward primer (IVTXgRNA_F). The 5′-end of the 20 bp target sequence

should be changed to $\boxed{\text{G}}$ for highly efficient transcription under the control of the T7 promoter.

Target sequence: 20 bp target (**Bold**) + PAM sequence (underlined)

$\boxed{\mathbf{G}}$**NNNNNNNNNNNNNNNNNNN**NGG

Primer set

IVTgRNA_F:

5' -GCGTTAATACGACTCACTATAG $\boxed{\mathbf{G}}$**NNNNNNNNNNNNNNNNNNN**-3'

IVTgRNA_R:

5′-AAAAGCACCGACTCGGTGCC-3′

2. Amplify the template DNA for in vitro transcription. Add the following to a 0.1 mL plastic tube on ice: 0.2 ng of template gRNA cloning vector, 2.5 μL of 10 μM forward primer (final, 0.5 μM), 2.5 μL of 10 μM reverse primer (final, 0.5 μM), 10 μL of 5X Q5 Reaction Buffer, and 0.5 μL of Q5 Hot Start High-Fidelity DNA Polymerase. Adjust volume to 50 μL with nuclease-free water. Perform PCR on a thermal cycler under the following conditions: 98 °C for 30 s; 20–25 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 2 min. Analyze 3 μL of PCR products by agarose gel electrophoresis (*see* **Note 2**).
3. Purify PCR products using the QIAquick PCR Purification Kit.
4. Perform in vitro transcription using the MEGAshortscript T7 Kit. Add the following to a 0.1 mL plastic tube on ice: 100 ng of purified PCR products, 2 μL of T7 10X Reaction Buffer, 2 μL of T7 ATP solution, 2 μL of T7 CTP solution, 2 μL of T7 GTP solution, 2 μL of T7 UTP solution, and T7 Enzyme Mix. Adjust volume to 20 μL with nuclease-free water.
5. Incubate the reaction at 37 °C for 2 h.
6. To remove the DNA template, add 1 μL of TURBO DNase, mix well, and incubate for 15 min at 37 °C.
7. Purify RNA using the MEGAclear Kit with RNase-free water.
8. The concentration of RNA can be determined by measuring the absorbance in a spectrophotometer at 260 nm, but we use the Qubit® Fluorometer because it provides a more sensitive estimate of the amount of RNA in solution. Store RNA at − 80 °C until use.

3.1.2 In Vitro Transcription of Cas9 RNA

1. We use vector pCAG-hCas9 as the template for in vitro transcription. Prepare the following primer set for in vitro transcription of hCas9.

   IVThCas9_F:

   5′- GCGTAATACGACTCACTATAGGGAGAATGGACAAGAAGTACTC-3′

   IVThCas9_R:

   5′-AGAGTCGCGGCCGCTCAC-3′
2. Amplify the template DNA for in vitro transcription. Add the following to a 0.1 mL PCR tube on ice: 0.2 ng of hCas9 expression vector, 2.5 μL of 10 μM forward primer (final, 0.5 μM), 2.5 μL of 10 μM reverse primer (final, 0.5 μM), 10 μL of 5X Q5 Reaction Buffer, and 0.5 μL of Q5 Hot Start High-Fidelity DNA Polymerase. Adjust volume to 50 μL with nuclease-free water. Perform PCR under the following conditions: 98 °C for 30 s; 20–25 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 4.5 min.
3. Purify PCR products using the QIAquick PCR Purification Kit or an alternative kit.
4. Perform in vitro transcription of hCas9 using the mMESSAGE mMACHINER T7 ULTRA Kit. Add the following to a 0.1 mL plastic tube on ice: 100–200 ng of purified PCR products, 2 μL of 10X T7 Reaction Buffer, 10 μL of T7 2X NTP/ARCA, and 2 μL of T7 Enzyme Mix. Make up to 20 μL with nuclease-free water.
5. Incubate the reaction at 37 °C for 2 h.
6. To remove the DNA template, add 1 μL of TURBO DNase, mix well, and incubate for 15 min at 37 °C.
7. For the poly(A) procedure, add the following reagents to the reaction mixture: 20 μL of 5X *E*-PAP Buffer, 10 μL of 25 mM $MnCl_2$, 10 μL of ATP Solution, and 36 μL of nuclease-free water.
8. Incubate at 37 °C for 30–45 min.
9. Purify RNA using the MEGAclear Kit with RNase-free water.
10. Measure the concentration of Cas9 RNA and store it at −80 °C until use.

3.2 Embryo Collection and Culture

1. Induce superovulation of female mice (*see* **Note 3**) by injecting 5 U (B6) or 7.5 U (B6D2F1) of PMSG, followed by 5 U (B6) or 7.5 U (B6D2F1) of hCG 48 h later (*see* **Note 4**). After injection of hCG, mate females overnight with males of the same strain. The following morning, check for the presence of a vaginal plug to determine the success of copulation (*see* **Note 5**).

2. On the morning of embryo collection, make several flat M16 drops (25 μL) on a 60 mm dish, and cover them with paraffin oil. Equilibrate M16 medium at 37 °C under 5% CO_2 in air.
3. At 21 h after hCG injection, sacrifice female mice and dissect out the oviducts.
4. Transfer the oviducts into a 35 mm Petri dish containing 1.5 mL of M2 medium at room temperature, and then remove the blood by shaking the dish.
5. Transfer all oviducts to a new 35 mm Petri dish containing 1.5 mL of M2-hyaluronidase.
6. Cut the oviduct ampulla using a 27-gauge hypodermic needle on a 1 mL syringe, and gently squeeze out the cumulus-oocyte complex into M2-hyaluronidase medium.
7. Incubate at 37 °C for several minutes until the cumulus cells fall off.
8. Collect the fertilized eggs using an embryo transfer pipet and transfer to a fresh dish containing M2 medium. After washing the eggs three times, culture in M16 medium at 37 °C under 5% CO_2 in air until microinjection.

3.3 Cytoplasmic Microinjection

The microinjection pipet for injecting Cas9/gRNA into eggs is made as shown in Fig. 1a.

3.3.1 Preparation of the Microinjection Pipet

1. Parameters such as filament temperature and pulling force should be optimized before the experiment. Pull thin-walled capillary tubes using a pipet puller (*see* **Note 6**).
2. Set a micropipet on the beveler at 35–40°, and grind the tip for 30 s.
3. To remove any residual particles on or in the pipets, wash them in ethanol while maintaining positive pressure using a syringe. Penetration of the micropipet can be confirmed by observing the release of small air bubbles from the pipet end.
4. Bend the tip of the pipet at 10–15° using a microforge.

3.3.2 Preparation of the Holding Pipet

The holding pipet for fixing the zygote in position to allow microinjection is made as shown in Fig. 1b.

1. Pull the non-filament glass tubes as described above for the microinjection pipets.
2. Set the pipet horizontally on the glass ball of the microforge, and cut the end at a width of 90–100 μm by flash-heating the microforge (*see* **Note 7**).
3. Set the pipet vertically on the microforge, and polish the broken end by careful heating.
4. Bend the tip of the pipet to an angle of 10–15° on the microforge.

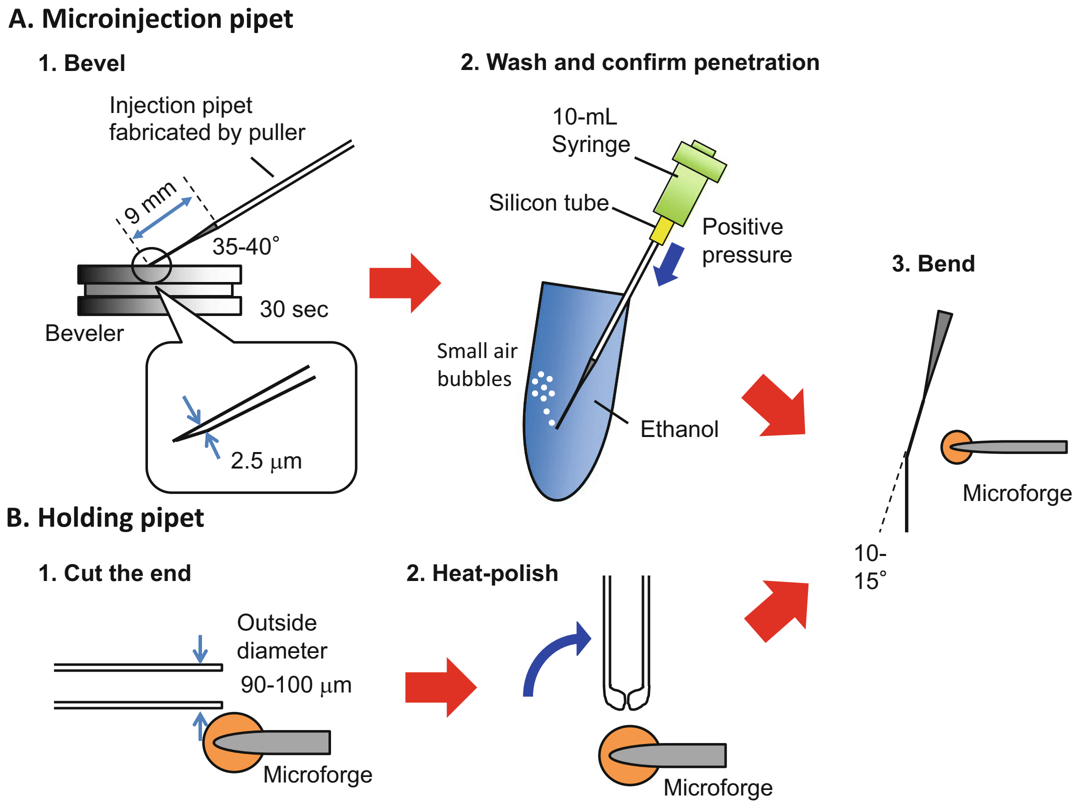

Fig. 1 Preparation of microinjection and holding pipets. (**a**) Microinjection pipet. (**b**) Holding pipet

3.3.3 Cytoplasmic Injection

1. Set up the micromanipulation system as shown in Fig. 2 (*see* **Note 8**).
2. Prepare both in vitro transcribed Cas9 mRNA and gRNA. For cytoplasmic injection of zygotes, 50–100 ng/μL of Cas9 mRNA and 10–50 ng/μL of gRNA are generally used. The Cas9/gRNA cocktail should be prepared just before microinjection or stored at −80 °C without repeated freezing and thawing (*see* **Note 9**).
3. We show the setup using the lid of a 100 mm plastic dish, which is used for Hoffman optics (*see* **Note 10**). Make several flat M2 drops (10–15 μL) on the center of the lid, and then place Cas9/gRNA drops (1 μL) to the lower right of the M2 drops (Fig. 2), and cover them with paraffin oil.
4. Set up an injection chamber on the microscope stage. Insert both holding and injection pipets into pipet holders at a slight angle (10–15°) to allow the tips to reach the bottom of the dish (Fig. 2).

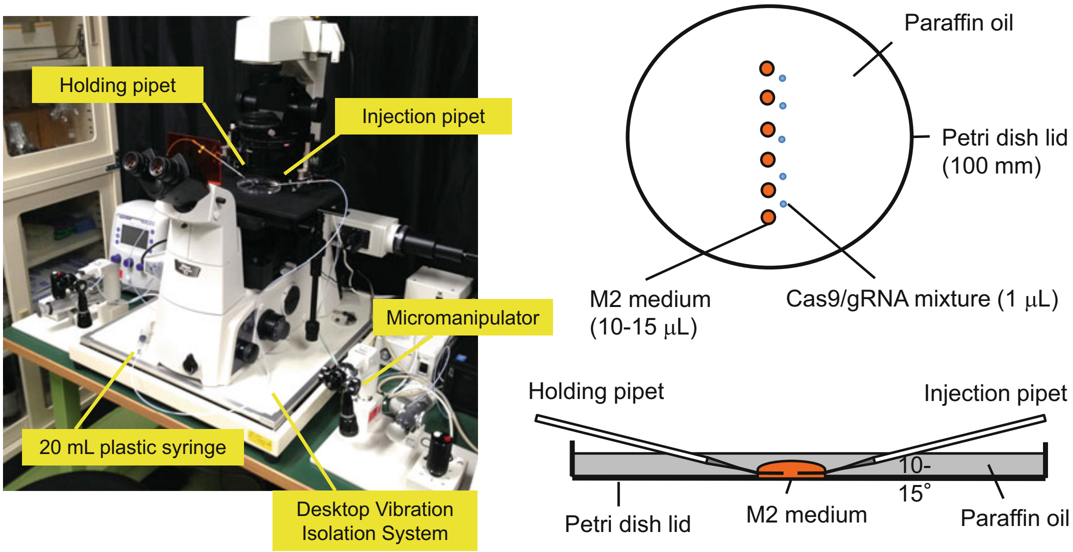

Fig. 2 Arrangement of microscope, micromanipulators, and injection chamber for cytoplasmic injection

5. Move the tip of an injection pipet into a Cas9/gRNA drop, and fill the tip with Cas9/gRNA by maintaining negative pressure on the syringe for a few minutes.
6. Move the tip of the injection pipet into the paraffin oil, and firmly push the piston of the syringe to confirm that the injection pipet is not closed (Fig. 3a, arrow).
7. Using an embryo transfer pipet, transfer 20–30 eggs into the M2 drop in the chamber.
8. Place the holding pipet next to an egg, and apply gentle vacuum to affix it to the tip of the pipet.
9. Keeping the holding pipet steady, adjust the focus of the microscope to confirm the position and number of pronuclei. Then, focus the microscope on the outline (plasma membrane) of the egg.
10. Squeeze firmly on the syringe just before injection to flush out M2 medium that may have entered the injection pipet, and maintain positive pressure.
11. Carefully insert the injection pipet into the cytoplasm, taking care not to damage the pronuclei (Fig. 3b) (*see* **Note 11**).
12. After successful penetration of the membrane, Cas9/gRNA will be spontaneously released into the cytoplasm of the egg because the injection pipet is kept at positive pressure. If the cytoplasmic granules around the tip of the pipet move, and obvious changes occur in the transparency of the surrounding region (Fig. 3c), you have successfully injected Cas9/gRNA (*see* **Note 12**).

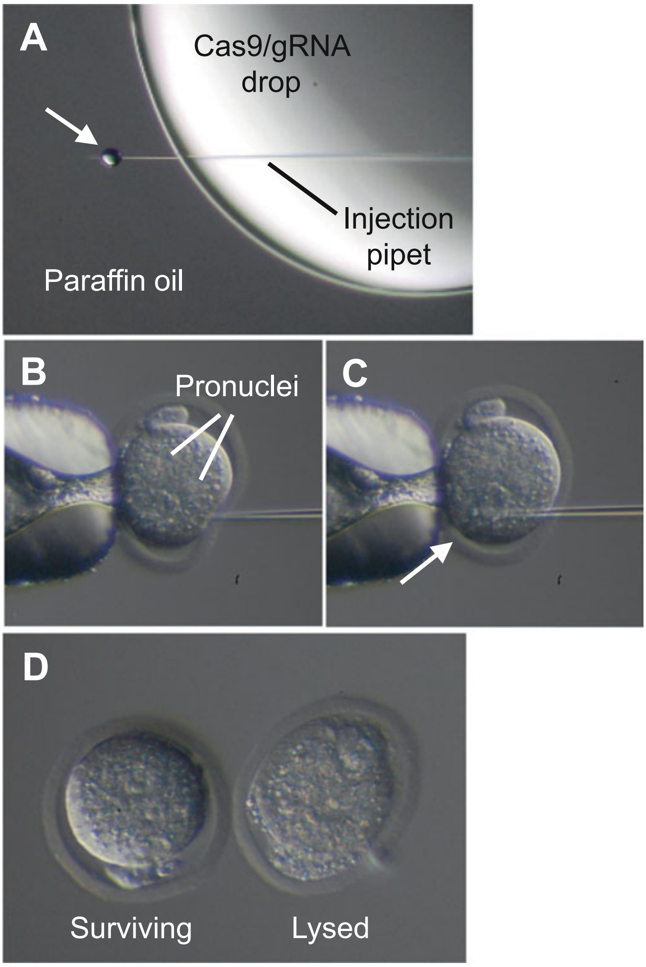

Fig. 3 Procedure for cytoplasmic injection. (**a**) Make sure that the injection pipet is not closed by confirming release of Cas9/gRNA buffer (arrow) from the end of the injection pipet. (**b**) Carefully insert the injection pipet into the cytoplasm, taking care not to hurt the two pronuclei. (**c**) If Cas9/gRNA is successfully injected into the cytoplasm, cytoplasmic granules around the tip of the pipet will move, and the transparency of the surrounding area will change (arrow). (**d**) Surviving eggs exhibit a distinct outline compared with lysed eggs

13. Once Cas9/gRNA is released, quickly pull the injection pipet out of the egg.
14. Release the egg from the holding pipet and place the injected egg into a separate area of the M2 drop.
15. Pick up a new egg and perform injection in the same manner. The injection pipet can be used continuously until (1) the injection pipet is unable to penetrate plasma membrane smoothly; (2) two eggs serially lyse immediately after injection; or (3) the injection pipet clogs or breaks.

16. When all the eggs in the drop have been injected, put them back into an M16 drop at 37 °C, and transfer new eggs into the M2 drop in the injection chamber.
17. After injection of all the eggs is completed, incubate them at 37 °C for an additional 30 min.
18. Separate surviving eggs from any that have lysed. Surviving eggs have a distinct outline compared with lysed eggs (Fig. 3d). Culture eggs in M16 medium at 37 °C under 5% CO_2 in air until embryo transfer or other manipulations.

3.4 Embryo Transfer

1. For production of mutant mice, transfer embryos from the 1-cell to morula stage into the ampulla of the oviduct (10–20 embryos per oviduct) of pseudopregnant foster females at 0.5 days post coitum (dpc) (*see* **Note 13**). Alternatively, blastocyst stage embryos can be transferred into the uterine horns of pseudopregnant females at 2.5–3.5 dpc.
2. Pups will be born at 19.5 dpc.
3. Extract genomic DNA from the tail tips of 3-week-old mice.

3.5 Assay for Genome Modification

1. To detect small genomic modifications, perform PCR analysis using primers flanking the targeted region. For the first screening, PCR products may be digested with a restriction enzyme that cleaves the Cas9 target site of the non-modified but not that of the modified genome (*see* **Note 14**). Analyze PCR products by gel electrophoresis or capillary and microchip electrophoresis.
2. Clone PCR products derived from mutant alleles into a TA-cloning vector and sequence eight or more clones of each mutant allele (*see* **Note 15**).

4 Notes

1. We purchase single gRNA (sgRNA) from Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA).
2. Optimum annealing temperature depends on the target sequence. If PCR products are not well amplified, change the annealing temperature.
3. We usually use B6 mice for strain establishment and B6D2F1 mice to determine the optimal experimental conditions. The main advantage of using hybrid mice is the high developmental rate of embryos both in vitro and in vivo.
4. In our lab, we inject ten females with PMSG and hCG at 3:00–4:00 PM (assuming a light period of 8:00 AM–8:00 PM).

5. Vaginal plugs should be checked in the early morning because they are expelled over time.
6. This microinjection pipet may be used without any modifications; however, further modifications such as micropipet beveling increases the survival rate of the eggs.
7. Be sure not to melt the pipet.
8. Pneumatic or oil microinjectors constructed with a glass or metal gasket syringe are typically used for holding and injection pipet control. Electric microinjectors, which enable the injection of very small volumes of liquid with great precision, are also available for injection pipet control. Alternatively, disposable plastic syringes can be used as the most economical choice. We usually use 10 mL plastic syringes for injection pipet control because high pressure can be rapidly delivered to the needle tip to drive DNA/RNA into the eggs.
9. RNA solution can be stored at −80 °C for up to 1 year. Do not repeat freezing and thawing, which promote degradation of RNA. It is also important to use RNase-free water to dilute the Cas9/gRNA mixture. However, do not use diethylpyrocarbonate (DEPC)-treated water, which is toxic to embryos.
10. Microinjection is generally performed using an inverted microscope with Hoffman modulation contrast optics or Nomarski differential interference contrast optics. A phase contrast microscope can also be used, but it is difficult to identify the location of pronuclei using this type of microscope. In general, Nomarski optics are used with a glass injection chamber. However, a recycled chamber is not recommended because it may be contaminated with RNase, which will degrade Cas9 mRNA and gRNA. By contrast, Hoffman optics can be used with disposable plastic dishes, which are free of RNase contamination.
11. In many cases, the injection pipet may be inserted into the egg boundary, where pronuclei are not located. However, cytoplasmic injection is more difficult than pronuclear injection because the plasma membrane is more elastic than the nuclear membrane and achieving penetration is more challenging. If the membrane cannot be penetrated, repeat the insertion a few times or change the insertion point. The use of injection pipets with sharp tips improves the success rate.
12. If apparent expansion of the egg is observed, the injection volume is too high.
13. It is better to transfer the manipulated embryos to oviducts as soon as possible after microinjection. Alternatively, embryos can be cultured until the blastocyst stage to determine the developmental rate in vitro or genome editing efficiency.

14. Alternatively, editing efficiency can be assessed using the surveyor nuclease assay, which uses enzymatic mismatch cleavage to detect single base mismatches or small insertions or deletions.
15. Occasionally, more than two mutant alleles are identified in an individual mouse. This is caused by mosaicism of somatic cells in mice generated by CRISPR-Cas9 injection [6]. Therefore, sequence analyses of mutant alleles should be performed using eight or more clones. In addition, the mutation profile of the germline of the founder (F0) mice is sometimes different from that of the tail tips used for genotyping; therefore, the sequences of the mutant alleles should be checked again in the F1 offspring.

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Chapter 7

Gene Targeting in Mouse Embryonic Stem Cells via CRISPR/Cas9 Ribonucleoprotein (RNP)-Mediated Genome Editing

Manabu Ozawa, Chihiro Emori, and Masahito Ikawa

Abstract

The CRISPR/Cas9-mediated genome-editing system enables the development of gene-modified mice using fertilized eggs. However, while the efficiency in developing gene knockout mice by inducing small indel mutations would be good enough, the successful ratio to create large side DNA knock-in (KI) by embryonic genome editing is still low. In contrast to the direct embryo KI method, gene targeting using embryonic stem cells (ESC) followed by chimeric mouse development by blastocyst injection still has several advantages, e.g., high-throughput in vitro targeting/screening or large-size DNA KI such as Cre, CreERT, TetON, and reporter fluorescent protein, or their fusion proteins can be carried out without serving animal lives. The ESC targeting can also be applied to strains such as BALB/c, of which embryos are known to be difficult to handle in vitro. This text describes the optimized method for either short- or large-size DNA KI in ESC by applying CRISPR/Cas9-mediated genome editing followed by chimera mice production to develop gene-manipulated mouse models.

Key words CRISPR/Cas9, Ribonucleoprotein, ESC, Knock-in, Chimera

1 Introduction

The generation of gene-modified mice followed by an analysis of their phenotype helps analyze specific gene functions or a detailed analysis of the physiological behavior of particular types of cells in the animal body. Such genetically engineered animal models have revealed numerous essential findings in the life science fields.

Gene manipulation of mouse embryos by CRISPR/Cas9 was first made by introducing single guide RNA and Cas9 mRNA into the pronucleus of fertilized eggs by micromanipulation [1]. Genome editing of mouse zygotes generally yields high efficiencies in indel mutations mediated loss-of-function, single nucleotide replacements, or short DNA fragment insertions using a single-strand oligonucleotide (ssODN) as a knock-in (KI) donor

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[2]. On the other hand, KI of large-size DNA cannot be accomplished in zygotes as efficiently as in the case of indel mutation. Thus, many zygotes for developing even one line of large-size KI animals are needed. From an animal welfare perspective, reducing the number of animals served for experiments is generally required. Furthermore, it is well known that some murine strains, such as BALB/c, which is a crucial strain for the immunology area, are susceptible to in vitro embryo handling, thus not suitable for direct zygote genome editing. Therefore, further improvement for developing gene-modified mice models is still strongly needed.

Conventional KI mouse development involves gene targeting embryonic stem (ES) cells, followed by blastocyst injection to create chimeras [3–5]. ESC targeting is thought to have several advantages compared to direct zygote genome editing for creating genetically engineered mouse models. For example, high-throughput genotyping screening can be performed in vitro, or the number of mouse lives used for a line development can be significantly reduced compared with direct embryo genome editing. However, the conventional KI efficiency of ESC is generally insufficient, even though a targeting vector contains 5′ and 3′ homologous arms and drug resistance gene cassettes for positive or negative selection because of the high frequency of random genomic integration. Therefore, a tune-upped method for improving gene targeting efficiency to ESC is needed.

This protocol describes a new ESC KI procedure in which efficiency is improved by using CRISPR/Cas9-mediated genome editing compared with the conventional targeting methods. Our method allows large DNA KI to ESC without a drug selection at acceptably high efficiency; thus, the vector construction procedures become much easier, and the cell culture period can be significantly shortened.

2 Materials

2.1 gRNA-Cas9 Ribonucleoprotein (RNP)

1. Gene-of-interest-specific crisprRNA: Alt-R CRISPR-Cas9 crRNA (IDT, Coralville, IA, USA).
2. Common tracrRNA: Alt-R CRISPR-Cas9 tracrRNA (IDT).
3. Cas9 nuclease: Alt-R S.p. Cas9 nuclease V3 (IDT).
4. RNA dilution buffer: Nuclease-Free Duplex Buffer (IDT).
5. Thermal cycler.

2.2 Single-Strand Oligonucleotides or Double-Strand DNA for Gene Knock-In

1. Single-stranded oligonucleotides (ssODN), length up to 250-base.
2. Cloning plasmid vector, e.g., pUC19.
3. Hi-fidelity DNA polymerase.

4. Specific PCR primers.
5. DNA cloning kit: In-Fusion HD Cloning Kit (Clontech).
6. Competent Cells, e.g., DH5α or JM109.
7. DNA purification kit.
8. Plasmid purification kit.
9. LB medium.
10. Ampicillin solution.

2.3 Embryonic Stem Cells, Doner Blastocyst, and Feeder Cells

1. Embryonic stem cell (JM8.A3 line from B6N [6], V6.5 line from B6-129 F1 [7], or B/C-a2 line from BALB/c (in house)).
2. Mouse embryonic fibroblast cells (MEF).
3. E3.5 blastocyst from B6 or ICR strain.
4. Embryo culture medium.
5. Embryonic stem cell medium (ESCM): Knockout-DMEM (Thermo Fisher) with 15% (v/v) fetal bovine serum, 2 mM GlutaMax, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM 2-mercaptoethanol, leukemia inhibitory factor, and t2i (0.2 μM PD0325901 and 3 μM CHIR99021)).
6. MEF medium: Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.
7. Gelatin coating solution: 0.1% gelatin in distilled water, autoclaved.
8. Gelatin-coated cell culture plate: Add a suitable volume of the gelatin coating solution to each well of the culture plate (1 mL for 6-well plate; 0.5 mL for 24-well plate; 100 μL for 96-well plate), and incubate for 2 h. Remove the solution, dry overnight, and store it at room temperature (RT).
9. Phosphate buffered saline (PBS) without calcium and magnesium.
10. Trypsin–EDTA solution: Dilute trypsin to 0.25% with 1 mM EDTA in PBS. Store at 4 °C.
11. Freezing medium: Bambanker (Nippon Genetics).

2.4 Electroporation System and Genomic DNA Preparation for PCR Genotyping

1. Neon Transfection System (MPK5000, Thermo Fisher).
2. Lysis buffer: Tail lysis buffer (#06169-95, Nacalai) with Proteinase K.
3. Phenol-Chloroform-Isoamyl Alcohol mixture solution.
4. DNA coprecipitation regent.
5. TA PCR cloning Kit or blunt-end PCR cloning kit (depending on the type of PCR polymerase used for genotyping).

2.5 Embryo Microinjection

1. Micromanipulator.
2. PVP medium: Desolve 12% (w/v) polyvinylpyrrolidone in HEPES-buffered (20 mM) KSOM at 4 °C overnight. Filter with a hydrophilic 0.2 μm PVDF membrane and store at −30 °C.

3 Methods

3.1 Targeting Vector Construction

1. Amplify a 1 kbp or longer fragment of 5′- or 3′-homology arms and KI cassette sequence by PCR. For In-Fusion DNA cloning, add 15-mer overlap sequences at the 5′ end of each PCR primer. Electrophorese PCR solution using agarose gel, cut the specific DNA band, and purify each amplified DNA fragment using a DNA purification kit.
2. Linearize backbone plasmid, e.g., pUC19, using an appropriate restriction enzyme that uniquely digests somewhere in the multi-cloning site for PCR fragments cloning. Electrophorese digested plasmid using agarose gel, cut the specific DNA band, and purify linearized plasmid using a DNA purification kit.
3. Ligate each PCR fragment, e.g., 5′-homology arm, 3-′-homology arm, and KI sequence simultaneously into the linearized cloning plasmid using In-Fusion HD Cloning Kit.
4. Transform competent cells using the fragment-cloned plasmid, plate them onto an LB plate containing ampicillin (100 μg/mL), and culture them overnight to select resistant clones.
5. Pick up several individual colonies, and culture them in liquid LB containing ampicillin (100 μg/mL) overnight at 37 °C with shaking.
6. Purify the plasmid the following day using a plasmid purification kit. Confirm the cloned sequence in the plasmid, i.e., targeting vector, by Sanger sequencing. Adjust the targeting vector concentration at 1 μg DNA/μL using nuclease-free water, and store at −30 °C until use.

3.2 Cas9-RNP Complex Preparation

1. TracrRNA and crisprRNA annealing: Dissolve tracrRNA and crisprRNA in Duplex Buffer (IDT, 200 μM each). Mix 2.2 μL tracrRNA and 2.2 μL crisprRNA with 5.6 μL Duplex buffer by gentle tapping, and anneal each RNA using a thermal cycler at 95 °C for 10 min followed by −1 °C/min stepdown cycles until 25 °C. Annealed RNA can be stored at −30 °C for 1 year.
2. Cas9-RNP complex formation: Incubate 1.25 μL of annealed RNAs with 0.75 μL Cas9 nuclease and 0.5 μL Neon R buffer (Neon Transfection System, Thermo Fisher) at 37 °C for 20 min to form Cas9-RNP complex. The Cas9-RNP complex can be stored at 4 °C for 1 month or at −80 °C for 2 years in single-use aliquots.

3.3 MEF Preparation

1. Mouse embryonic fibroblast (MEF) development: Euthanize a pregnant female (14.5-day postcoitum). Dissect the uterus with fetuses, and place them in a 100 mm culture dish with PBS. Release the fetuses and placentas from the uterus. Remove heads and internal organs from all fetuses. Mince the fetuses with a pair of scissors, and dissociate cells with 0.25% trypsin-EDTA solution (about four fetuses/mL) for 5–10 min at 37 °C. After inactivating the trypsin by adding ten times the volume of MEF medium, pipette well, and seed a cell suspension in a dish (about four fetuses/10 cm dish). Change culture medium after 4–5 h post-seeding to remove unattached cells, and let MEF cells grow to ~90% confluence.
2. Collect growing MEF by trypsin treatment, and wash once with MEF medium by centrifugation (280 × *g*, 5 min). Resuspend precipitated MEF pellet in Bambanker (all cells in 100 mm dish in 500 μL of Bambanker per one cryotube), freeze them using an −80 °C freezer overnight, and then store them in liquid nitrogen.
3. Mitotically inactivating MEF: Thaw a tube of frozen MEF (**step 2** in Subheading 3.3), and seed them into four 100 mm dishes. Expand the MEF to a confluent state. Irradiate proliferating MEF with X-rays (50 Gy) to halt the cell cycle. Trypsinize irradiated MEF for 5 min and collect in a new tube. Wash MEF by fresh MEF medium by centrifugation (280 × *g*, 5 min), and resuspend in Bambanker (1.5×10^6 in 500 μL/tube). Cryopreserve MEF as mentioned above (**step 2** in Subheading 3.3), and store in liquid nitrogen until use.

3.4 Gene Targeting of ESC and PCR Genotyping

1. ESC culture: Seed X-ray-irradiated mitotically inactivated MEF (**step 3** in Subheading 3.3) on a gelatin-coated 60 mm dish (1.5×10^6 cells for two 60 mm dishes) at least 1 day before ESC seeding.
2. Thaw a frozen ESC stock tube (2×10^5 ESC in 500 μL Bambanker/tube) quickly using a 37 °C water bath, and resuspend ESC in 5 mL ESCM. Mix well, centrifuge at 280 × *g* for 5 min, and remove the supernatant. After suspending the ESC palette with ESCM, seed 1×10^5 ESC onto a 60 mm dish containing pre-seeded MEF, and incubate at 37 °C with 5% CO_2.
3. Preparation of Cas9-RNP and DNA KI donor solution: Mix the following materials in the same 1.5 mL tube: 10 μL of Neon R buffer, 1 μL of Cas9-RNP complex (**step 2** in Subheading 3.2), and 1 μL of ssODN (200 pmol/μL) or 1 μL of plasmid-targeting vector (1 μg/μL). The total amount of Cas9-RNP mixture is 12 μL. Keep Cas9-RNP mixture on ice until electroporation.

4. Neon Transfection System is used for electroporation of ESC (Fig. 1a, b). For electroporation, use ESC at ~50% confluency (2–3 days after the ESC seeding, *see* **Note 1**).
5. Wash culturing ESC once with PBS, then treat with 800 uL of trypsin-EDTA solution, and incubate for 5 min at 37 °C for enzymatic digestion. Add 1 mL of ESCM and dissociate ESC into a single cell by gentle pipetting. Centrifuge ESC-containing solution for 5 min at 280 × *g*, discard supernatant, resuspend ESC in a fresh 1 mL ESCM, and count the cell numbers. Transfer 1×10^5 ESC to a new 1.5 mL tube, and wash them three times with PBS.
6. After the final centrifugation, resuspend ESC in a 12 μL Cas9-RNP mixture (**step 3** in Subheading 3.4), and mix well by gentle pipetting. Serve resuspended ESC for electroporation using the Neon Transfection System. The Neon Transfection System uses a single pulse at 1400 V and 30 ms for Cas9-RNP transfection.
7. Culture electroporated ESC in ESCM with MEF. Change medium every day.
8. Three days after the electroporation, digest ESC by trypsin-EDTA, and passage them at 1×10^3 ESC cells/60 mm dish containing ESCM and feeder MEF. Change medium every day.
9. Five to 7 days after the passage, aspirate medium from ESC culture dish and replace with PBS. Pick up single ESC colonies with 5 μL of PBS using a 20 μL pipette. Place the colonies in a round-bottom 96-well plate which contains 15 μL of trypsin-EDTA solutions. Keep the 96-well plate on ice until 48 individual colonies are picked-up. Then, incubate the plate for 5 min in a 37 °C humid 5% CO_2 incubator. Add 80 μL of ESCM and dissociate cells by gentle pipetting.
10. Seed the 40 μL of ESC suspension into a gelatin-coated feeder-free 96-well plate for PCR genotyping and the 60 μL of ESC suspension into a well in a 24-well plate with feeder MEF for making frozen ESC stock.
11. For making frozen stock of ESC clones, culture the 24-well plate at 37 °C, 5% CO_2 until ESC reaches ~80% confluency. Add 100 μL of trypsin-EDTA, and culture for 5 min at 37 °C to digest ESC into single cells. Add 500 μL of ESCM, and dissociate cells by gentle pipetting, wash them by centrifugation, and resuspend the ESC in 1.5 mL Bambanker solution. Stock one clone into three aliquot tubes (500 μL/tube), and place the tubes at −80 °C overnight. Then, store frozen ESC in liquid nitrogen until use.

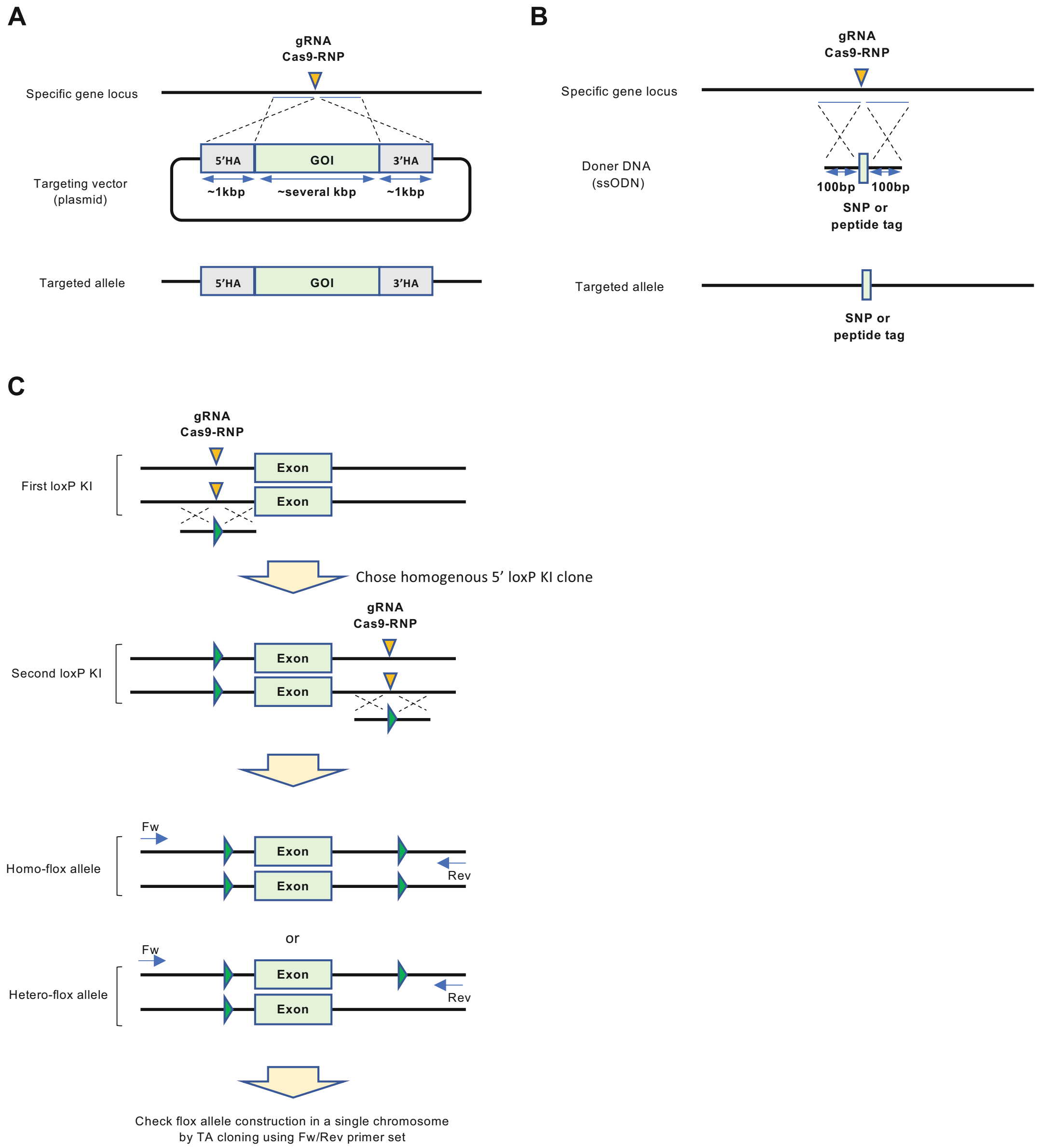

Fig. 1 CRISPR/Cas9 ribonucleoprotein (RNP)-mediated DNA fragment integration into ESC genome. (**a**, **b**) Schematic representations of DNA fragment KI strategies. Plasmid vector containing GOI cassette flanked by approximately 1 kbp homology arms (**a**) or ssODN containing short DNA sequences such as SNPs or peptide tag and flanked by approximately 100-base homology arms (**b**) is transduced into ESC via electroporation with site-specific CRISPR/Cas9 RNP. (**c**) Schematic representation of flox allele construction. ssODN containing 5′ loxP flanked by approximately 100-base homology arms is first transduced into ESC via electroporation with site-specific CRISPR/Cas9 RNP. After genotyping for 5' loxP KI screening, second electroporation using 3′ loxP ssODN is transduced into ESC clones with 5′ loxP KI allele. Finally, flox allele construction is confirmed by PCR using Fw/Rev. primer set followed by the PCR fragment cloning to a sequencing plasmid for checking whether both loxP are KI-ed in the same chromosome

12. For DNA preparation of PCR genotyping, culture the 96-well feeder-free ESC-containing plate at 37 °C, 5% CO_2 until ESC reaches ~90% confluency.
13. Remove ESCM from each well, wash twice with PBS, add 100 μL lysis buffer (**step 2** in Subheading 2.4), mix well, and transfer to a new 1.5 mL tube. Incubate ESC lysate at 65 °C for at least 1 h to extract genomic DNA.
14. Add 100 μL PCI, mix well by vigorous shaking, and centrifuge 14,000 × *g* for 10 min at 4 °C. Transfer 80 uL of supernatant aqueous phase to a new 1.5 mL tube. Add appropriate volume of DNA coprecipitation regent, and mix well by vortexing. Add 200 μL 100% ethanol and mix well by vortexing. Centrifuge the DNA solution at 14,000 × *g* for 10 min at RT. Discard the supernatant carefully so as not to lose the precipitated DNA palette. Add 500 μL of 70% ethanol to the tube, and wash the DNA palette by gentle inverting. Centrifuge the tube at 14,000 × *g* for 5 min at RT. Discard the supernatant carefully so as not to lose the precipitated DNA palette.
15. Leave the tube on the table for a few minutes to dry the DNA pellet, dissolve it in DNase-free water, and measure the DNA concentration.
16. Perform genomic PCR using locus-specific primer sets to amplify the target region. Check the sequence, and choose the ESC clones having the desired mutations to produce chimeric mice.

3.5 Flox Allele Generation

1. To generate a floxed allele, use two individual ssODN with loxP sequence flanked by 100-base of 5′ or 3′ homology arms (Fig. 1c).
2. Introduce 5′ side loxP ssODN to ESC, and check its integration as described in Subheading 3.4.
3. Repeat the KI procedure using 3′ side loxP ssODN into the 5′ loxP-targeted ESC clones.
4. Amplify ESC genomic region using a primer set flanking 5′ and 3′ loxP sequence by PCR (Fig. 1c).
5. Clone the PCR fragment to TA cloning plasmid (in the case of using Adenine-overhung PCR polymerase) or blunt-end cloning plasmid (in the case of using blunt-end PCR polymerase).
6. Transform competent cells by using the cloned plasmid, amplify cells, purify the plasmid, and check the sequence whether both 5′ and 3′ loxP sequences are targeted into the same chromosome.

3.6 Preparation of ESC for Blastocyst Injection

1. Seed X-ray-irradiated mitotically inactivated MEF on a gelatin-coated 60 mm dish (1.5×10^6 cells for two 60 mm dishes) at least 1 day before seeding ESC. Thaw a tube of ESC frozen stock (**step 11** in Subheading 3.4), seed them onto a gelatin-coated 60 mm dish with the feeder MEF layer, and incubate them at 37 °C, 5% CO_2.
2. Let ESC grow to 60–70% confluence (usually takes 2–3 days). On the morning of the injection day, trypsinize ESC, resuspend in 10 mL ESCM, and pass them to a gelatin-coated feeder-free 100 mm dish. Incubate them at 37 °C for 30 min to let MEF settle and attach to the bottom of the dish. Collect the supernatant in which ESC is concentrated into a 15 mL tube, and centrifuge at 280 × *g* for 5 min. Suspend the ESC pellet with 1 mL ESCM containing HEPES (20 mM). Keep ESC on ice until the blastocyst injection.

3.7 Preparation of Blastocyst and Microinjection of ESC

1. Use ICR strain blastocyst as a recipient for JM8.A3 or V6.5 ESC or B6 strain blastocyst as a recipient for B/C-a2 ESC injection for distinguishing chimerism by coat color (Fig. 2).
2. Inject 5 IU PMSG in the abdominal cavity of adult ICR or B6 females. After 48 h, inject 5 IU hCG into the same female, and mate the hormone-treated females with adult same strain males. Check the copulatory plug in the vagina the following day (embryonic day 0.5, ED0.5).
3. At ED1.5, collect the ampulla and place them in KSOM drops. Recover the two-cell stage embryo by flushing the oviduct with KSOM using a flushing needle inserted into the infundibulum. Wash the collected embryos with fresh KSOM using a mouth pipette, and freeze them by vitrification according to the CARD protocol (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/ebvitri.html), and store them in liquid nitrogen until use.
4. Thaw frozen two-cell stage embryos 2 days before the ESC injection following the CARD protocol (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/index.html). Culture the embryos in 50 μL of KSOM drop covered with mineral oil in 37 °C, 5% CO_2 incubator for 2 days to let them develop to the blastocyst stage.
5. Prepare glass pipettes for embryo holding and ESC injection. Prepare a micromanipulator equipped with these pipettes.
6. Dispense 10 μL drops of PVP medium (**step 2** in Subheading 2.5) onto a 60 mm dish, and cover the drops with mineral oil. Place embryos in a PVP medium drop and add 1~3 μL of the ESC suspension.
7. Wash the inside of an injection pipette with PVP medium, and pick up five to ten round-shaped ESC using the injection

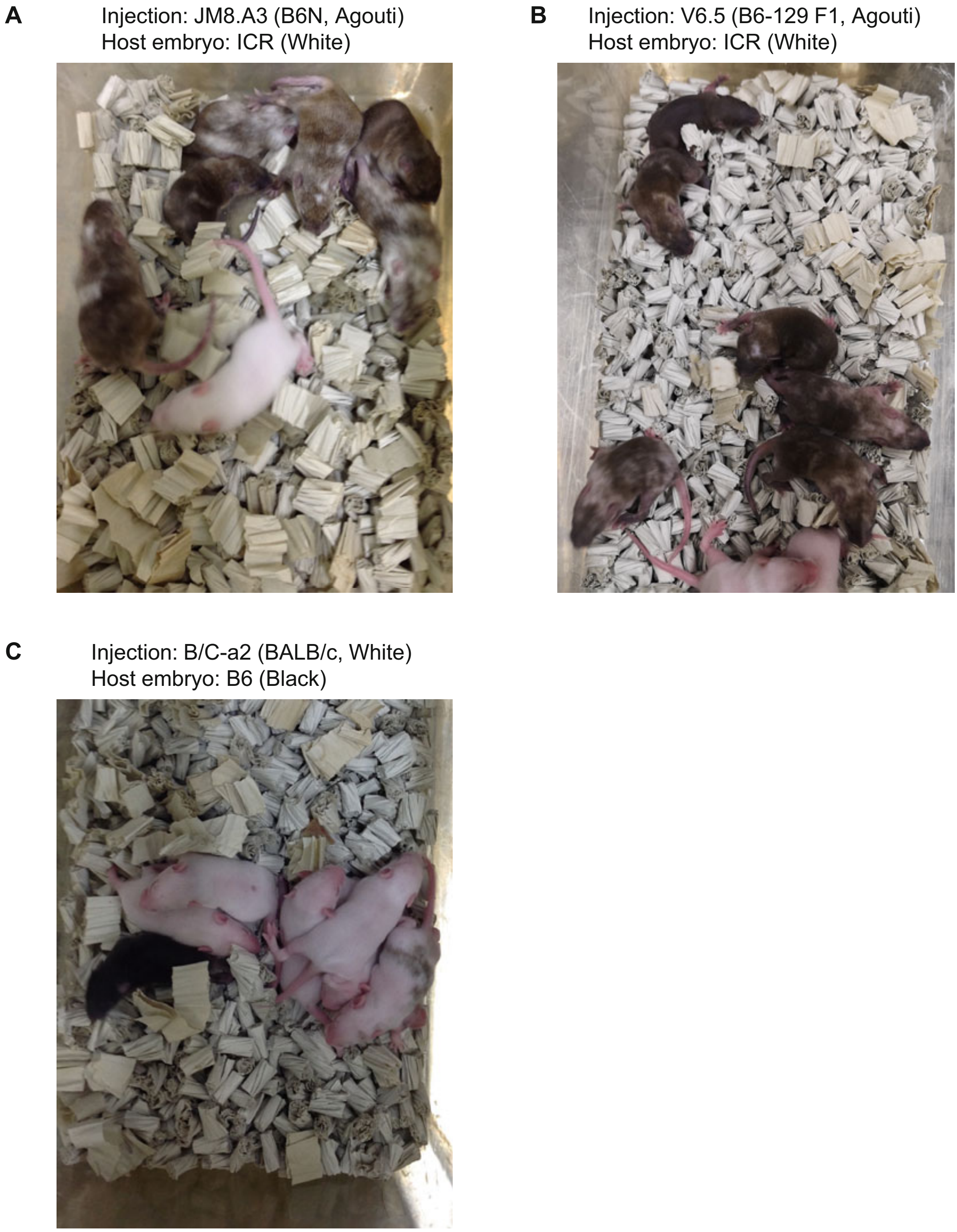
A
Injection: JM8.A3 (B6N, Agouti)
Host embryo: ICR (White)
B
Injection: V6.5 (B6-129 F1, Agouti)
Host embryo: ICR (White)
C
Injection: B/C-a2 (BALB/c, White)
Host embryo: B6 (Black)

Fig. 2 Representative images of chimera mice. (**a**, **b**) ESC derived from JM8.A3 ESC line (C57BL/6N, agouti hair) (**a**) or V6.5 (B6 129 F1, agouti hair) are injected into ICR (albino, white hair) blastocysts followed by embryo transfer to mother surrogates. (**c**) ESC derived from BALB/c embryo (albino, white hair) are injected into C57BL/6J (black hair) blastocysts followed by embryo transfer to mother surrogates

pipette (*see* **Note 2**). Hold a blastocyst, and make a hole in the zona pellucida with a piezoelectric pulse. Expel ESC in the blastocoel and pull the pipette out of the blastocyst.

8. Wash the injected blastocyst with KSOM, and incubate them in a 50 μL KSOM drop covered with mineral oil at 37 °C, 5% CO_2 until all the blastocyst injections are done. Transfer the injected blastocyst into the uterus of pseudopregnant female mice (2.5-day postcoitus) (20 injected blastocysts per head).
9. Once the embryo-transferred surrogate mother has given birth, select at least two male chimeras with the highest possible chimerism (70% or higher as confirmed by coat color, *see* **Note 3**), and cross them with wild-type females to obtain heterozygous knock-in F1 individuals. Afterward, breed KI individuals with appropriate partners to obtain specific genotypes or genetic backgrounds suitable for the specific research.

4 Notes

1. The growth speed of ESC differs among various strain backgrounds or clones. It is better to examine the growth speed of ESC lines before starting any experiments.
2. Round ESC should be selected for injection. As the incubation time of ESC at RT increases, the cells become more angular. It is best to inject 20~30 embryos in one injection cycle.
3. The chimera contribution rate of the injected ESC varies significantly among the ESC clones or ESC strain backgrounds. If high chimerism individuals are not obtained, additional blastocyst injection should be performed using other clones.

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Chapter 8

Generation of Knock-In Mouse by Genome Editing

Wataru Fujii

Abstract

Knock-in mice are useful for evaluating endogenous gene expressions and functions in vivo. Instead of the conventional gene-targeting method using embryonic stem cells, an exogenous DNA sequence can be inserted into the target locus in the zygote using genome-editing technology. In this chapter, I describe the generation of epitope-tagged mice using engineered endonuclease and single-strand oligodeoxynucleotide through the mouse zygote as an example of how to generate a knock-in mouse by genome editing.

Key words Knock-in mice, Genome editing, Engineered endonuclease, Single-strand oligodeoxynucleotide (ssODN), Zygote

1 Introduction

Genetically modified mice are useful for functional analysis of genes or genome sequences in vivo. Knock-in technology can accurately delete endogenous genome regions or mimic spontaneous mutations, such as single-nucleotide polymorphisms (SNPs), by target nucleotide substitution. In addition, knock-in mice incorporating the reporter gene (fluorescent protein, LacZ, synthetic epitope-tag, or other marker genes) can be used to observe endogenous gene expressions in vivo. Further, knock-in animals are used for conditional gene modification via recombination reactions such as the Cre-loxP system.

A conventional knock-in method, gene targeting, uses a DNA donor template, which has flanking DNA of the target locus together with knock-in sequences. The donor DNA is recombined with the endogenous homologous sequence; then, the designed sequence is inserted into the target locus. This process depends on a DNA repair system, a homologous recombination reaction [1–3]. However, homologous recombination-mediated modification is inefficient in vertebrate cells, except for some cell lines, such as DT40 cells [4, 5]. Therefore, accurately modified cells are selected

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from a large amount of the starting cell pool via drug selection. This method cannot be applied to mammalian zygotes because a large number of the zygotes cannot be prepared and handled at one time. Pluripotent stem cells (such as embryonic stem cells) or cultural male germline stem cells (known as GS cells) are usually used for homologous recombination-mediated gene targeting and generation of knock-in mice [6–8]. However, these multistep experiments (expansion of drug-selected genome-modified cells, generation of chimera/transplanted mouse, and generation of its next generation) are time-consuming, expensive, and laborious.

In order to overcome these limitations, a new method that can directly modify the gene in zygotes has been developed using genome-editing technology. Genome editing uses the engineered endonuclease, which induces DNA double-strand breaks (DSBs) at the target locus and increases intracellular DNA repair activity. Genome-editing technology can efficiently produce knockout and knock-in cells, even from a limited number of zygotes [9, 10]. Although the conventional method needs to wait for the sexual maturation of chimeras or transplanted animals before generating the next generation, genome editing via zygotes can produce knock-in mice in one generation.

Various types of DNA templates have been used as knock-in donors. Commercially available single-strand oligodeoxynucleotides (ssODNs), procurable quickly and inexpensively, are widely used as knock-in template DNA [11–22]. The use of ssODNs simplifies the generation of knock-in mice because the construction of the conventional targeting plasmid vectors is complicated and time-consuming. By microinjection of ssODN and engineered endonuclease into the zygotes, various kinds of knock-in mice incorporating exogenous sequences can be generated (restriction endonuclease sites [13–15], nucleotide substitution [13, 16, 17], a loxP sequence [18, 19], and a short amino acid epitope-tag sequence [19, 20]). As well as the short insertions, longer sequences, such as fluorescent reporter genes or Cre recombinase, can be inserted by using a long single-strand DNA (lsDNA) as a knock-in template. The lsDNAs can be obtained from suppliers or self-synthesizing [21, 22]. Adeno-associated virus (AAV) carries a vector based on a single-stranded DNA, which can also be used as a knock-in template in zygotes. Recombinant AAV serotype six [23, 24] and serotype one [25] efficiently transduce into mouse zygotes and could be used as a knock-in template [24, 25]. Double-strand DNA or plasmid DNA can also be used for genome editing in zygotes, but its knock-in efficiency is limited [18, 19]. A recent report suggests that the cell cycle stage of used zygotes affects knock-in efficiency using dsDNA [26].

In this chapter, I describe a method for generating epitope-tag knock-in mice by the microinjection of ssODN together with engineered endonuclease into mouse zygotes as an example of a

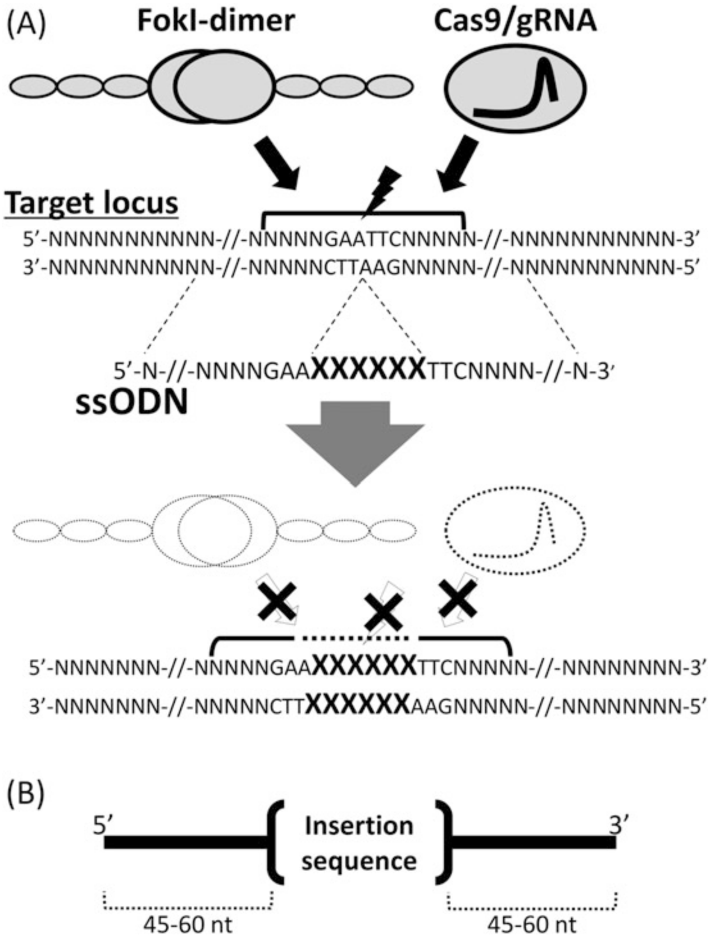
(A)
FokI-dimer
Cas9/gRNA
Target locus
5′-NNNNNNNNNNN-//-NNNNNGAATTCNNNNN-//-NNNNNNNNNN-3′
3′-NNNNNNNNNNN-//-NNNNNCTTAAGNNNNN-//-NNNNNNNNNN-5′
5′-N-//-NNNNGAAXXXXXXTTCNNNN-//-N-3′
ssODN
5′-NNNNNNN-//-NNNNNGAAXXXXXXTTCNNNNN-//-NNNNNNNN-3′
3′-NNNNNNN-//-NNNNNCTTXXXXXXAAGNNNNN-//-NNNNNNNN-5′
(B)
5′
Insertion sequence
3′
45-60 nt
45-60 nt

Fig. 1 The knock-in strategy by genome-editing technology. (**a**) Schematics of knock-in using ssODN and genome editing. Knock-in sequence (indicates as "**XXXXXX**") of ssODN is designed at the target locus of engineered endonucleases (FokI-dimerized ZFN/TALEN pairs or Cas9/gRNA complex). The modified allele should be designed so as not to be recognized by the endonucleases. (**b**) The ssODN used for zygote-mediated knock-in together with the endonucleases. Two homology arms (45–60 nt) are added to each side to flank the desired insertion or mutation (indicates as **Insertion sequence**)

knock-in method (Fig. 3). Using this method, I have generated knock-in mice with more than 40% [15, 16]. Almost all of the procedures are the same as those for generating knockout mice by genome editing. The most important step in the zygote-mediated generation of knock-in mice using genome-editing technology is to design the target sequence of the engineered endonuclease and the targeting template DNA (Fig. 1).

2 Materials

Other than for the ssODN preparation, all materials used are the same as the materials used for the generation of knockout mice by cytoplasmic microinjection described in Chap. 5 (*see* **Note 1**).

2.1 ssODN

1. ssODN: Purchased as a lyophilized product. High-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE)-purified ssODNs are better, but reverse-phase cartridge purification is usable.
2. RNase- and DNase-free distilled water: Used for the dilution of nucleotides.
3. Cas9 mRNA and gRNA: 10–100 ng/microL and 10–20 ng/microL (or ZFN/TALEN pair: 5–10 ng/microL each).

3 Methods

3.1 Design of ssODN as Knock-In Targeting DNA

The knock-in template ssODN sequence should be designed so as not to be recognized by the engineered endonuclease. If the DNA sequence of the knock-in allele would be digested by the endonuclease, knock-in and indel would be induced simultaneously in the target locus (Fig. 1a). The target sequence of the endonuclease should be designed to overlap the target locus. The length of each homology arm of ssODN is about 45–60 nt (Fig. 1b) (*see* **Notes 2** and **3**).

3.1.1 For Nucleotide Substitution

The ssODN sequence and the target locus of the endonuclease should be designed carefully for the induction of nucleotide substitution by the genome editing, because the substituted nucleotide sequence is not conspicuously different from the wild-type sequence (Fig. 2). In the cases of ZFN and TALEN, the alteration site should be designed on the endonuclease recognition DNA sequence (Fig. 2a). If it is designed at the interval of the recognition sequences, the random indel may be induced together with the precise nucleotide substitution (Fig. 2b). In the case of CRISPR/Cas, even if several mismatched nucleotides exist within the recognition sequence of gRNA, the modified sequence is recognizable as a target locus of the Cas9/gRNA complex (Fig. 2d). Therefore, the ideal design for the target nucleotide site is to be on the protospacer adjacent motif (PAM) of gRNA so that the modification disrupts the PAM sequence (Fig. 2c).

If such a design cannot be applied to the target nucleotide site, it is effective to use ssODNs which have the target substitution together with synonymous substitutions in which the endonucleases are not recognizable [27]. Note that the codon change

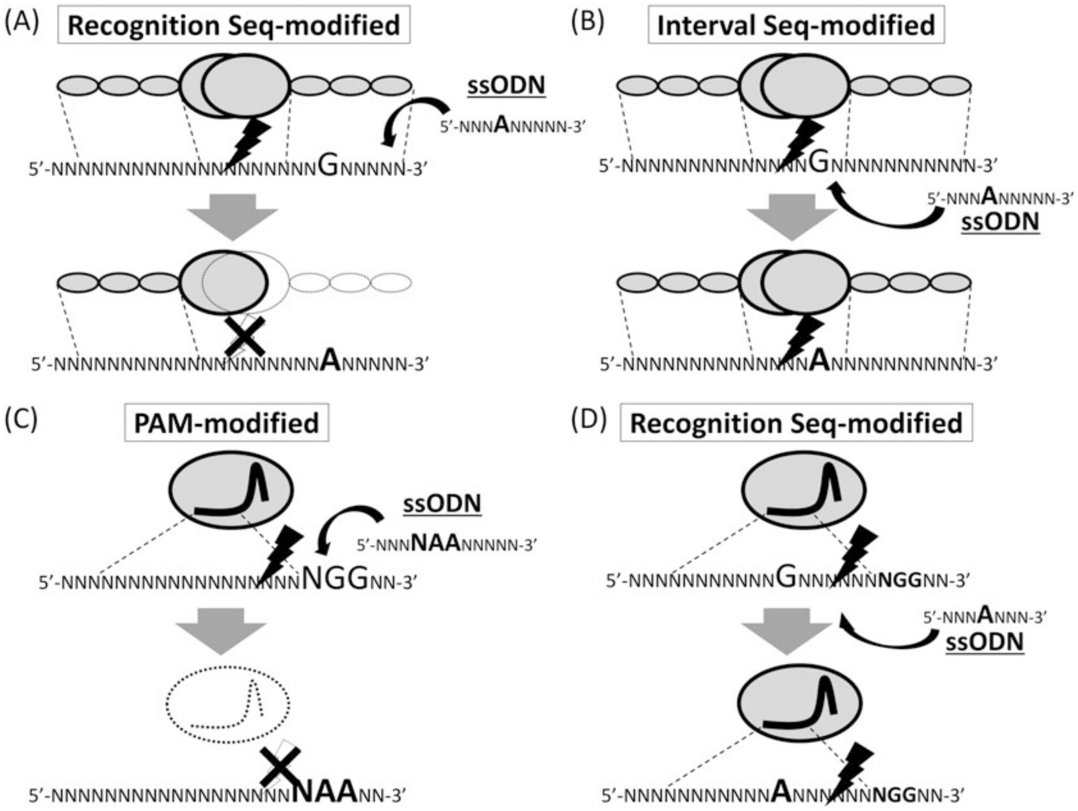
(A)
Recognition Seq-modified
ssODN
5′-NNNANNNNN-3′
5′-NNNNNNNNNNNNNNNNNNNNGNNNNN-3′
5′-NNNNNNNNNNNNNNNNNNNANNNNN-3′
(B)
Interval Seq-modified
5′-NNNNNNNNNNNNNNGNNNNNNNNNNNN-3′
5′-NNNANNNNN-3′
ssODN
5′-NNNNNNNNNNNNNNANNNNNNNNNNNN-3′
(C)
PAM-modified
ssODN
5′-NNNNAANNNNN-3′
5′-NNNNNNNNNNNNNNNNNNNNGGNN-3′
5′-NNNNNNNNNNNNNNNNNNNAANN-3′
(D)
Recognition Seq-modified
5′-NNNNNNNNNNNGNNNNNNNNGGNN-3′
5′-NNNANNN-3′
ssODN
5′-NNNNNNNNNNNANNNNNNNNGGNN-3′

Fig. 2 The design of the nucleotide substitution using ssODN and the engineered endonucleases. (**a**, **b**) The nucleotide substitutions using ZFN or TALEN. When the substitution is introduced to the sequence recognized by nuclease, the modified allele cannot be digested by the same endonucleases again (**a**). In the case of the substitution to the interval of recognition sequence, FokI can digest the modified allele again, and undesired indel can be induced to the locus simultaneously (**b**). (**c**, **d**) The nucleotides substitution using CRISPR/Cas. The modified allele is not recognized by the Cas9-gRNA complex by introducing the substitution on the PAM (**c**). On the other hand, the introducing the substitution to the target sequence of the gRNA can cause re-recognition of the same Cas9/gRNA (**d**)

may affect the stability of the RNA and the translation efficiency. Orthologous CRISPR-derived Cas9 [16, 28–30] and synthetic mutant Cas9 [31, 32] recognize the nonconventional sequence as a PAM, so these can be used to design knock-in at the untargetable locus by conventionally used *S. pyogenes* Cas9. In addition, Cas9 nickase [20, 33], FokI-Cas9 [34, 35], or high-fidelity Cas9 mutants [32, 36] can be used to expand the target locus for knock-in.

3.1.2 For Target-Insertion of Short-Epitope Sequence

In the case of the knock-in of a short-epitope-tag sequence such as FLAG or HA, the design of endonuclease is easier than the nucleotide substitution, because the DNA sequence of the modified locus becomes much different from that of the unmodified wild-type. As with nucleotide substitution, the target sequence of endonucleases and ssODN should be designed so as not to be recognized by the

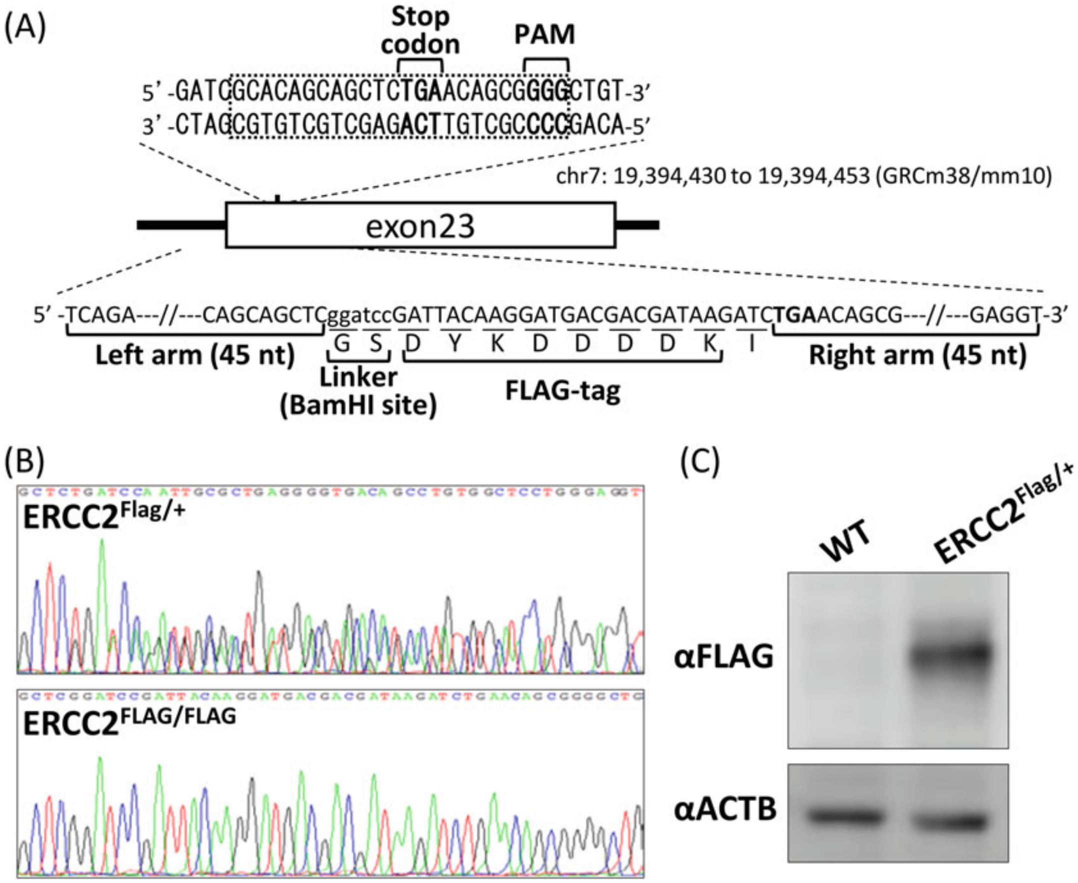
(A)
Stop codon
PAM
5’ -GATCGGCACAGCAGCTCTGAACAGCGGGGCTGT-3’
3’ -CTAGCGTGTCGTCGAGACTTGTCGCCCCGACA-5’
chr7: 19,394,430 to 19,394,453 (GRCm38/mm10)
exon23
5’ -TCAGA---//---CAGCAGCTCggatccGATTACAAGGATGACGACGATAAGATCTGAACAGCG---//---GAGGT-3’
Left arm (45 nt)
G S D Y K D D D D K I
Linker (BamHI site)
FLAG-tag
Right arm (45 nt)
(B)
GCTCTGATCCA ATTGCGCTGAGGGGTGACA GCCTGTG GCTCCTGGGA GGT
ERCC2Flag/+
GCTCGGATCCGATT ACAAGGATGACGACGATAAGAT CTGAACAGCGGGGGCTG
ERCC2FLAG/FLAG
(C)
WT
ERCC2Flag/+
αFLAG
αACTB

Fig. 3 An example of knock-in mice generation. (**a**) Schematic illustration of the ERCC2 gene structures, sequences around the target locus (upper), and ssODN template (lower). Black-dot box on the sequence around the target locus indicates the PAM and the sequence recognized by the Cas9-gRNA complex. The target sequence contains the stop codon of Ercc2. (**b**) A waveform data of genome sequence of a heterozygous (upper) or a homozygous (lower) knock-in pup. These data were obtained by direct sequencing of PCR amplicons. (**c**) Immunoblotting of wild-type and tagged mouse. Tail-tip-derived proteins of wild-type (WT) and of heterozygous (ERCC2$^{Flag/+}$) pups were immunoblotted with anti-FLAG antibody and anti-βActin antibody

endonucleases after modification. The tag sequence can be designed at the gap site as well as at the endonuclease recognition sites in the cases of ZFN and TALEN, because the distance between each of the endonuclease pairs is important for FokI endonuclease activity. For CRISPR/Cas-mediated tagging, the recognition sequence of gRNA or PAM should overlap with the tag insertion locus (*see* **Note 4**).

Figure 3 shows an example of a FLAG-tag knock-in in the C-terminal region of the mouse Ercc2 gene introduced by using CRISPR/Cas. Guide-RNA was designed on the stop codon of Ercc2, which is expected not to recognize the target locus after precise modification (Fig. 3a) (*see* **Note 5**).

3.2 Preparation of Injection Nucleotides

The preparations of Cas9 mRNA and gRNA (or other engineered endonuclease RNAs) are the same as described in other chapters.

3.2.1 Dilution of the Purchased ssODN

1. Spin down the dry-storage oligodeoxynucleotides using a desktop centrifuge.
2. Add nuclease-free distilled water to adjust the concentration to 1 mg/mL.
3. Vortex for 10 s.
4. Incubate the ssODN solution at 37 °C for 30 min.
5. Vortex for 10 s.
6. Aliquot for 10 microL and store at −20 °C until use.

3.2.2 Preparation of Injection Solution

1. Cryopreserved ssODN and in vitro transcribed RNA are thawed at room temperature.
2. Mix them with nuclease-free distilled water to adjust the concentrations as follows:

   Cas9 mRNA and gRNA: 10–100 ng/microL and 10–20 ng/microL(or ZFN/TALEN pair: 5–10 ng/microL each)

   ssODN: 100–200 ng/microL
3. Centrifuge for few minutes at room temperature just before injection.

3.3 Zygote Collection, Microinjection, Embryo Transfer, and Genotyping

These procedures are the same as the methods used for the generation of knockout mice (*see* Chap. 5) (*see* **Notes 6** and 7).

4 Notes

1. Although pronuclear microinjection can be used as well as cytoplasmic microinjection for the generation of knock-in mice, cytoplasmic microinjection results in a higher incidence of embryo development after the injection [37]. Instead of microinjection, the electroporation-mediated transduction of ssODN together with engineered endonucleases could be used for the generation of knock-in mice as well [38, 39].
2. It was reported that the combination of the strand of gRNA and ssODN affected the knock-in efficiency in culture cells [40]. Either the same or different combination of the strand of gRNA and ssODN worked in mouse zygotes.

3. A previous report suggests that the most suitable base lengths of the homology arms were 18 and 20 nt in the zebrafish zygote [41] but were inefficient in the mouse zygote under our experimental conditions. In culture cells, 60–90 nt of homology arms would be better than a length of 30 nt [42]. If the knock-in efficiency is low, the extension of each of the homology arms may increase the knock-in efficiency in mouse zygotes.
4. Although a single loxP sequence can be inserted using ssODN, simultaneous cis knock-in of each loxP sequence to generate “floxed” allele (two-donor method) is inefficient via zygotes [43]. Instead of the two-donor method, sequential knock-in at one- and two-cell stages can generate floxed alleles through preimplantation embryo development [44].
5. Genome PCR-direct sequencing is usually used for genotyping genome editing alleles. However, bulk PCR amplicons would contain the various indel-induced amplicons, making it difficult to precisely detect the presence of knock-in sequence from waveform data of the direct sequencing (Fig. 3b). PCR-restriction fragment length polymorphism (PCR-RFLP) can help screen the knock-in allele. To use PCR-RFLP for the genotyping, the ssODN should be designed as the restriction enzyme site is created or eliminated after the knock-in. For the knock-in of an epitope tag sequence, the restriction enzyme site can be designed between the open reading frame of the target gene and the epitope sequence. When positioned as shown in Fig. 3b, the enzyme site can function as a screening marker for PCR-RFLP and as a linker sequence that does not affect the conformation of the target gene or the interaction of target genes with other molecules. I usually apply the BamHI recognition sequence (5′-GGATCC) because it encodes glycine-serine, which is generally used as an amino acid linker (Fig. 3b).
6. For the efficient generation of knock-in zygotes, it is essential to prepare engineered endonucleases that efficiently introduce DSB into the target locus. Suppose the generation efficiency of knockout mice using genome-editing technology in your laboratory is lower than is generally reported, the used constructs, equipment, or experimental conditions should be considered to optimize. The injecting amount of the solution into the cytoplasm seems to vary among researchers, resulting in the different knock-in efficiencies even using the same concentration of the solution. The condition of the needle also affects the injecting amount. Cytoplasmic microinjection is more challenging to adjust the injecting amount of the solution than pronuclear microinjection. Confirming the injecting process by well-trained researchers would be helpful. Our microinjection condition is shown as a movie file [45].

7. Several studies have reported that nonhomologous endo-joining (NHEJ) inhibitors such as SCR7 increase the knock-in efficiency when using ssODNs in mouse zygotes [46, 47]. However, the efficiencies of the uninhibited experiments in these reports are lower than the efficiency described in general reports [46, 47]. Therefore, it is unclear how effective NHEJ inhibitors are for knock-in using ssODN in zygotes.

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Chapter 9

Introduction of Genetic Mutations Into Mice by Base Editor and Target-AID

Hiroki Sasaguri

Abstract

Generating genetically modified animal models that precisely recapitulate disease characteristics forms an integral and indispensable tool to understanding disease pathophysiology. Recently, important advances in genome editing technologies have enabled us to efficiently create sophisticated animal models in short periods of time. Base editing is a modified CRISPR/Cas system that induces base substitution at targeted genomic regions. Here I describe a basic protocol to introduce disease-relevant pathogenic mutations into mice utilizing two representative base editing tools, Base Editor and Target-AID.

Key words CRISPR/Cas, Base editing, Base Editor, Target-AID, Point mutations, Single-nucleotide polymorphism (SNP), APOBEC1, PmCDA1

1 Introduction

The generation of genetically modified animal models that precisely recapitulate disease characteristics is critically important for disease pathophysiologies to be understood and for therapeutic strategies to be developed. Previously, transgenic mouse models that overexpress disease-relevant molecules were mainly used to reproduce pathologies in vivo [1]. Although in many cases these mice exhibit the pathological hallmarks of specific diseases in question, it is becoming clear that the overexpression paradigm often causes artificial phenotypes in those models that are not related to the diseases of interest. This is due to intrinsic drawbacks of the overexpression paradigm such as the random insertion of transgenes into the genome and unphysiological expression patterns [2, 3]. On the other hand, gene knock-in strategies enable disease characteristics to be recapitulated in an accurate manner [4]. This process, however, is time-consuming and requires enormous effort to generate knock-in mice by conventional gene-targeting methods due to the low efficiency of homologous recombination in embryonic stem

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(ES) cells. The development of the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)-mediated knock-in strategy dramatically improved the efficiency of mutant mouse generation because this system can efficiently trigger genomic modifications at target sites determined by single-guide RNAs (sgRNAs) [5]. In addition, CRISPR/Cas can edit the genome directly in the zygotes of desired mouse strains. These embryos can then be transferred to foster mothers to obtain founder (F0) mutant mice, although mosaicism often occurs in F0 mice because the mutation takes place at some point after creation of the zygote.

New genome editing technologies have been developed by modifying CRISPR/Cas systems [6]. Base Editor (BE) and Target-AID are engineered proteins composed of deactivated Cas9 fused with cytidine deaminases [7, 8]. BE is a fusion protein of *Streptococcus pyogenes* Cas9 (SpCas9) and rat APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 1), while Target-AID (activation-induced cytidine deaminase) is composed of SpCas9 and sea lamprey PmCDA1 (Petromyzon marinus cytosine deaminase 1). These base editing tools convert C:G base pairs to T:A at target sites by a deamination reaction with a reduced rate of indel formation because deactivated Cas9 or Cas9 nickase is used in the system. Here, I introduce a basic protocol to generate mutant mice by microinjection of base editing tool mRNA and sgRNAs into mouse zygotes [9].

Protocols of mouse embryo manipulation in this article except for microinjection of base editing tools are developed in the Division of Reproductive Engineering, Center for Animal Resources and Development (CARD) at Kumamoto University (Professor Toru Takeo). I recommend visiting the homepage and seeing the details including valuable pictures and videos (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/onlinemanual.html).

2 Materials

Currently, there is a huge repertoire of base editing tools available to the research community [10]. In addition to the original BE and Target-AID tools, adenine base editors (ABEs) that can convert G:C pairs to A:T pairs and glycosylase base editors (GBEs) that can substitute C:G pairs to A:T pairs or A:T pairs to G:C pairs [11] are also available. Users need to first select an appropriate tool to introduce desired mutations according to sgRNA sequence, presence of the protospacer adjacent motif (PAM) sequence, and their active windows [10]. Most of the vector plasmids for these base editing tools are available from Addgene (https://www.addgene.org/).

2.1 PCR to Prepare Templates for In Vitro Transcription

1. Base editing vectors: The Addgene catalog numbers of the BE3 and Target-AID vectors composed of Cas9n (D10A Cas9), uracil glycosylase inhibitor (UGI), and cytidine deaminases (rat APOBEC1 for BE3 and sea lamprey PmCDA1 for Target-AID) are #73021 (pCMV-BE3) and #79620 (pcDNA3.1_pCMV-nCas-PmCDA1-ugi pH1- gRNA (HPRT)), respectively.
2. Primers: Prepare primers as follows.

   T7-BE3-F; GCCGCTAATACGACTCACTATAG.

   T7-BE3-R; GCGGGTTTAAACTCAATGGT.

   T7-Target-AID-F; GAACCCACTGCTTACTGGCT.

   T7-Target-AID-R; GTGGAATCGAAATCTCGTGAT.

   sgRNA-F; taatacgactcactata XXXXXXXXXXXXXXXXXXXX gttttagagctagaa.

   sgRNA-R; AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC.

   For the sgRNA-F primer, put the sgRNA target sequence in "X" without the PAM sequence. The targeted "C" should reside between the fourth and eighth bases for BE3 and second to fourth bases for Target-AID sgRNA sequences, respectively. The addition of one or two "G" in front of the target sequence may be required for efficient in vitro transcription (*see* **Note 1**). Dissolve primers with distilled water or Tris-EDTA (TE) buffer to a final concentration of 10 μM.
3. PCR polymerase: Prepare PCR polymerase with high fidelity.
4. Micropipettes.
5. Thermal cycler.
6. Gel electrophoresis apparatus.
7. 1% Tris-acetate-EDTA (TAE) agarose gels for BE and Target-AID template PCR products and 2% TAE agarose gels for sgRNA template PCR products.
8. PCR purification kit.
9. Ultraviolet-visible spectrophotometer for measurement of DNA concentration.

2.2 In Vitro Transcription of BE and Target-AID mRNA

1. Clean gloves and masks.
2. Nuclease-free water, micropipette tips, and tubes.
3. RNase decontamination solution.
4. Micropipettes.
5. T7 RNA polymerase kits for BE and Target-AID mRNAs and sgRNAs.
6. DNase.
7. Poly(A) tailing kit.

8. RNA purification kit.
9. Thermal cycler.
10. Gel electrophoresis apparatus.
11. 1% agarose-formaldehyde gels for BE and Target-AID mRNA and 2.5% agarose-formaldehyde gels for sgRNAs.
12. Ultraviolet-visible spectrophotometer for measurement of RNA concentration.

2.3 In Vitro Fertilization

1. PMSG (Pregnant Mare's Serum Gonadotropin) (37.5 IU/mL in sterile saline).
2. hCG (human chorionic gonadotropin) (37.5 IU/mL in sterile saline).
3. 1 mL disposable syringe.
4. Preincubation medium such as FERTIUP® (COSMO BIO).
5. Fertilization medium such as CARD MEDIUM® (COSMO BIO).
6. Modified human tubal fluid (mHTF) medium.
7. Micropipettes.
8. Pipette tips for preparation of dishes.
9. Pipette tips for insemination.
10. Plastic dishes (35 mm × 10 mm).
11. Fine scissors.
12. Pair of watchmaker's #5 forceps.
13. Micro-spring scissors (5 mm blade).
14. Dissecting needle.
15. Filter paper.
16. Glass capillaries for embryo handling.
17. Microscope.
18. Humidified incubator (37 °C, 5% CO_2, 95% air).

2.4 Microinjection to Mouse Zygotes

1. HTF medium.
2. 37 °C, 5% CO_2 incubator.
3. Male mice (C57Bl/6J, ≥10 weeks old) for collection of sperm.
4. Female mice (C57Bl/6J, 26–30 days old) for collection of ova.
5. Pregnant mare serum gonadotropin (PMSG hormone).
6. Human chorionic gonadotropin (hCG hormone).

2.5 Embryo Transfer Into the Oviduct

1. Female mice on Day 1 of pseudopregnancy (the day on which a vaginal plug is observed).
2. Micro-spring scissors (5 mm blade).
3. Pair of watchmaker's #5 forceps.

4. Serrefine clamp.
5. Wound clip and clip applicator.
6. Plastic dish (35 mm × 10 mm).
7. Glass capillaries for embryo transfer and handling.

2.6 Genotyping of Blastocysts and Mice

1. Lysis buffer for blastocysts: 100 mM Tris–HCl, 100 mM KCl, 0.02% gelatin, 0.45% Tween 20, 60 μg/mL yeast tRNA, and 125 μg/mL proteinase K.
2. Lysis buffer for mouse tails:10 mM Tris–HCl (pH 8.5), 5 mM EDTA (pH 8.0), 0.2% SDS, 200 mM NaCl, and 20 μg/mL proteinase K.
3. Primers: Prepare primers that are appropriate for Sanger sequencing. Primers can be designed using web tools such as Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/).
4. PCR polymerase.
5. Sequencing reagent.
6. Thermal cycler.
7. Purification reagent after sequencing pre-reaction.
8. DNA sequencer.

2.7 Analysis of Off-Target Mutations

1. Prediction of off-target sites: Off-target sites can be predicted using web tools such as Cas-OFFinder (http://www.rgenome.net/cas-offinder/) or COSMID (https://crispr.bme.gatech.edu/). Design primers for PCR and target sequencing in the same way as for genotyping.
2. Sequencing: The same as in Subheading 2.6.

3 Methods

3.1 Preparation of BE3 and Target-AID mRNAs

1. Perform PCR reaction as per manufacturer's instructions using template DNA (BE3 or Target-AID vectors) and primers prepared in Subheading 2.1.
2. Run a 1% agarose gel to confirm presence of appropriate PCR products (Fig. 1a).
3. Purify the PCR products. For final elution, use nuclease-free water to avoid contamination of RNase. Measure the concentration of purified DNA with an ultraviolet-visible spectrophotometer.
4. Perform in vitro transcription as per the manufacturer's instructions (*see* **Note 2**).
5. Incubate the reaction mixture with DNase.

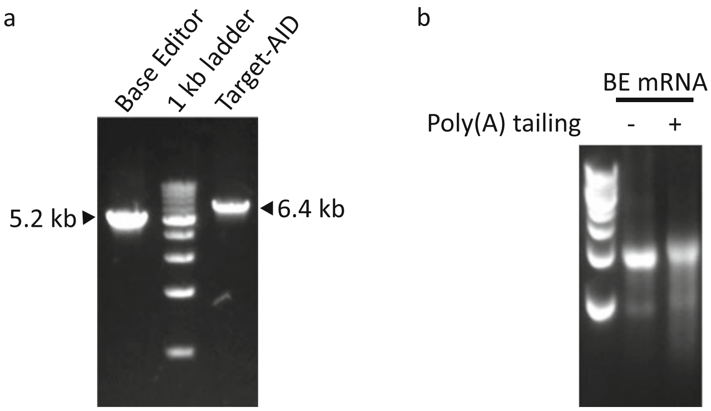

Fig. 1 In vitro transcription of BE and Target-AID mRNA. (**a**) PCR for preparation of templates for in vitro transcription of BE3 or Target-AID mRNAs. PCR products of BE and Target-AID are 5.2 kb and 6.4 kb, respectively. (**b**) Electrophoresis of BE mRNA after in vitro transcription and addition of poly(A) tail. If addition of poly (A) tails is successful, there will be a shift of bands

6. Take small amount of mixture and stock as a negative control (no poly(A) tail) in step 8.
7. Add poly(A) tail.
8. Run a 1% agarose-formaldehyde gel to confirm presence of appropriate mRNA with poly(A) tails. If addition of poly (A) tails is successful, there will be a shift of bands (Fig. 1b).
9. Purify the mRNA with RNA purification kit according to manufacturer's instructions.
10. Measure the concentration of mRNAs by an ultraviolet-visible spectrophotometer, and confirm that the A260/A280 nm signal ratio is around 2.0. Make aliquots and stock at −80 °C (*see* **Note 3**).

3.2 Preparation of sgRNAs

1. Perform PCR reaction as per manufacturer's instructions.
2. Run a 2% agarose to confirm presence of appropriate PCR products (Fig. 2a).
3. Purify the PCR products. For final elution, use nuclease-free water to avoid RNase contamination. Measure the concentration of purified DNA with an ultraviolet-visible spectrophotometer.
4. Perform in vitro transcription following the manufacturer's instructions (*see* **Note 2**).
5. Treat the reaction mixture with DNase.
6. Purify with RNA purification kit according to the manufacturer's instructions.

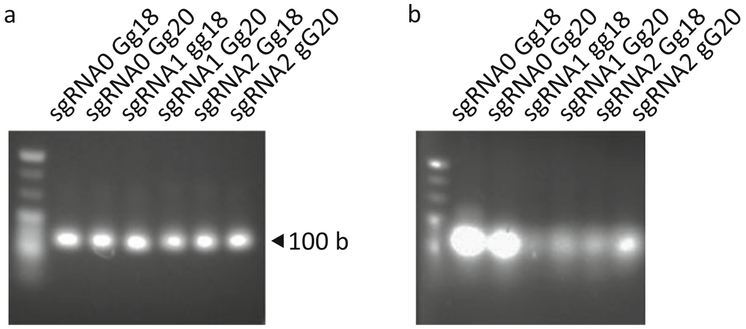

Fig. 2 In vitro transcription of sgRNAs. (**a**) PCR for preparation of templates for in vitro transcription of sgRNAs. PCR products are approximately 100 bp. (**b**) Electrophoresis of sgRNAs after in vitro transcription

7. Run a 2.5% agarose-formaldehyde gel to confirm the presence of appropriate sgRNAs (Fig. 2b).
8. Measure the concentration of sgRNAs with an ultraviolet-visible spectrophotometer, and confirm that the A260/A280 nm signal ratio is around 2.0. Make aliquots and stock at −80 °C.

3.3 In Vitro Fertilization

(See details at http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/mouseivf.html.)

1. Induce superovulation by injecting 7.5 IU of PMSG i.p. into each mature female mouse (8–12 weeks old). PMSG is usually administered during the light-on cycle, between the hours of 14:00 and 18:00.
2. Follow this up 48–52 h later with a 7.5 IU i.p. injection of human chorionic gonadotropin (hCG).
3. Prepare dishes as per the instructions below, and keep them in an incubator (37 °C, 5% CO_2 in air) to allow them to gas-equilibrate.
4. Put one drop (100 μL/drop) of preincubation medium into a dish, and cover with liquid paraffin 30 min before collecting sperm. Place the dish in an incubator.
5. Put one drop (200 μL/drop) of fertilization medium into a dish, and cover with liquid paraffin for 10 min before collecting oocytes. Place the dish in an incubator (*see* **Note 4**).
6. Put four drops (80 μL/drop) of mHTF into a dish and cover with liquid paraffin. Place the dish in an incubator and incubate for at least 30 min.
7. Sacrifice one or two mature male mice (3–6 months old), and remove the cauda epididymis, avoiding contamination by fat, blood, and tissue fluid as much as possible.

8. Place the tissue on sterile filter paper to blot away any blood and fluid.
9. Place the removed cauda epididymis in a sperm dish containing liquid paraffin.
10. Cut the duct of each cauda epididymis using a pair of microspring scissors, and then use a dissecting needle to gently press the surface of the cauda epididymis to release the sperm within.
11. Use a dissecting needle to introduce the clots of spermatozoa released from the cauda epididymis into the drop of preincubation medium (*see* **Note 5**).
12. Allow the sperm to capacitate by placing the suspension in an incubator (37 °C, 5% CO_2 in air) for 60 min before insemination.
13. Sacrifice a superovulating mature female mouse (8–12 weeks old) approximately 15–17 h after administering hCG.
14. Dissect the mouse to expose the abdominal cavity.
15. Move the digestive tract from inside the abdomen and expose the uteri, oviducts, and ovaries.
16. Remove the uteri, oviducts, and ovaries, and place them on sterile filter paper.
17. Remove the oviducts (ampullae) only, avoiding as much as possible the collection of any fat, blood, and tissue fluid. Immerse the removed oviducts in liquid paraffin in a fertilization dish.
18. Use forceps to hold the oviduct against the base of the fertilization dish, and then use a dissecting needle to tear open the ampulla of the oviduct and release the cumulus-oocyte-complexes (COCs) from within. Drag them into the drop of fertilization medium (200 μL) (*see* **Note 6**).
19. Place the fertilization dish with COCs in an incubator (37 °C, 5% CO_2 in air) for 30–60 min before the insemination step.
20. Use the tip of a pipette (Pipette Tip Cat.No.114; Quality Scientific Plastics) to add appropriate amounts (usually about 3 μL) of sperm suspension to the drop of fertilization medium containing the COCs.
21. Place the fertilization dish in an incubator (37 °C, 5% CO_2 in air).
22. Three hours after the insemination step, wash the oocytes three times in fresh mHTF (80 μL) in a washing dish, avoiding the transfer of fertilization medium.
23. Six hours after insemination, observe the oocytes in the third drop of mHTF, and remove any parthenogenetic oocytes which have only one pronucleus (*see* **Notes** 7 and **8**).

24. After overnight culture of the oocytes, transfer only the two-cell stage embryos obtained to the fourth drop of mHTF in the washing dish. These embryos can be vitrified, transferred to recipient females, or cultured to the blastocyst stage.

3.4 Freezing Fertilized Embryos

In the event that users prefer to perform microinjection at a later date, follow the protocol below to freeze the fertilized embryos.

1. Prepare drops of 1 M DMSO as follows: A large drop (~500 μL) and equal numbers of small drops (50 μL) to the number of cryotubes that the user intends to prepare (115 embryos per one tube).
2. Six hours after insemination, transfer the fertilized embryo with two pronuclei to the large drop.
3. Transfer 115 embryos from the large drop to each small drop.
4. Transfer the 115 embryos from one small drop with 5 μL DMSO to a cryotube.
5. Place the cryotube on a pre-chilled (0 °C) cool block bath.
6. Repeat **steps 3–5** for the remaining embryos.
7. Five minutes after the last transfer of embryos, gently add 45 μL of pre-chilled (0 °C) preservative solution (DAP213) to each tube along with the tube wall.
8. Five minutes after the addition of DAP213, place the tubes on a storage cane pre-chilled in liquid nitrogen, and immediately put into a liquid nitrogen refrigerator. At this step, loosen the cryotube caps to allow the liquid nitrogen to enter the cryotubes.
9. On the day of microinjection, take the cryotubes from the liquid nitrogen refrigerator, remove the caps, discard the liquid nitrogen inside the tubes, and place them at room temperature for 30 s.
10. Add 0.9 mL of pre-warmed (37 °C) 0.25 M sucrose into each tube, and pipette ten times over a period of 30 s.
11. Transfer the embryos to a dish. Add another 0.4~0.5 mL of 0.25 M of sucrose to each tube and collect the remaining embryos.
12. Transfer the embryos to an 80 μL drop of KSOM embryo medium, and incubate for 10 min in a 37 °C incubator.
13. Wash the embryos twice by transferring them to the second and third drops of KSOM, and incubate until microinjection.

3.5 Microinjection of Cytoplasm of Mouse Zygotes to Evaluate Efficiency at Blastocyst Stage (See Note 9)

1. Thaw the RNA aliquot solutions of BE or Target-AID mRNA and sgRNAs on ice.
2. Prepare the RNA solution on ice as follows (*see* **Note 10**):

   Base editing tool mRNA, x μL (200 ng/μL)

   sgRNA, y μL (60 ng/μL)

   Nuclease-free water, $40 - (x + y)$ μL
3. Centrifuge the RNA solution at 9100 × g for 30 min at 10 °C.
4. Take 20 μL of supernatant.
5. Transfer zygotes with two pronuclei to the drop and place on the stage of inverted microscope.
6. Hold one zygote with holding pipette and move the tip of microcapillary closer to the zygote.
7. Inject 100–150 pl of RNA solution into cytoplasm.
8. Repeat **steps 1**–**3** for the remaining zygotes (*see* **Note 11**).
9. Incubate the zygotes for 72 h until the blastocyst stage.

3.6 Genotyping at Blastocyst Stage

1. Collect blastocysts 72 h after microinjection.
2. Extract genomic DNA from blastocysts by incubating in 10 μL of lysis buffer for each blastocyst following the protocol below:

   56 °C for 10 min

   95 °C for 10 min
3. Perform PCR using adequate primer sets for the target region (*see* **Note 12**).
4. Run a gel to confirm the presence of appropriate PCR products.
5. Perform Sanger sequencing according to the manufacturers' instructions (*see* **Note 13**).
6. Select the best injection condition and proceed to Methods in Subheading 3.7.

3.7 Embryo Transfer into the Oviduct

(See details at http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/transfer.html.)

1. Repeat the microinjection step (Subheading 3.5) with the best condition which is determined in the genotyping analysis of blastocysts (Subheading 3.6).
2. Incubate the zygotes overnight (approximately 20 h until embryo transfer), and select embryos that develop to two-cell stage.
3. Anesthetize a female mouse.
4. Expose the ovary, oviduct, and part of the uterine horn as per the conventional procedure.
5. Attach a Serrefine clamp to the fat pad on the ovarian bursa (*see* **Note 14**).

6. Observe the oviduct under a stereomicroscope, and confirm the position of the infundibulum and ampulla using forcep tips or by changing the position of the Serrefine clamp.
7. Position the oviduct by changing the orientation of the Serrefine clamp and the mouse (*see* **Note 15**).
8. Place a 200 μL droplet of KSOM/AA in a dish (without liquid paraffin), and introduce 20 embryos into the droplet.
9. Aspirate air and medium in alternate intervals of 2–3 mm into a glass capillary in preparation for embryo transfer. Draw ten embryos into the glass capillary (*see* **Note 16**).
10. Using a pair of watchmaker's #5 forceps and micro-spring scissors, make a slit in the wall of the oviduct between the infundibulum and ampulla.
11. Insert the tip of the capillary containing the embryos into the slit, and then push the capillary further up toward the ampulla.
12. Use the forceps to hold the portion of the oviduct into which the capillary was inserted.
13. Expel the embryos and 2–3 of the air bubbles into the ampulla (*see* **Note 17**).
14. Withdraw the capillary gently from the slit (*see* **Note 18**).
15. Push the ovary, oviduct, and uterine horn back into the abdomen, and close the wound using wound clips.
16. Repeat the process to transfer the remaining ten embryos into the other oviduct as described above.
17. Keep mice warm on a 37 °C warming plate until they recover from the effects of the anesthesia.

3.8 Genotyping of Mice

1. A section of mouse tail is cut (5~10 mm) and incubated overnight in lysis buffer at 55 °C. Genomic DNA is extracted by the conventional phenol-ethanol method.
2. PCR is performed using adequate primer sets. Use PCR polymerase that is suitable for crude samples.
3. Run a gel to confirm the presence of appropriate PCR products.
4. Perform Sanger sequencing according to the manufacturers' instructions.

3.9 Analysis of Off-Target Mutations

1. Predict off-target mutations that accept up to three mismatches by using web tools such as Cas-OFFinder (http://www.rgenome.net/cas-offinder/) or COSMID (https://crispr.bme.gatech.edu/).
2. Perform Sanger sequencing according to the manufacturers' instructions (*see* **Note 19**).

4 Notes

1. Users can remove the first or first and second base(s) when "G" is added in front of target sequences (G + 19 bases or GG + 18 bases). It is recommended to try several sgRNAs of different length (19–21 including added "G") and number of added "G" to select the sgRNA with the highest efficiency for each targeted mutation. This is done by analyzing the genomic DNA sequence at the blastocyst stage (*see* Subheading 3) before proceeding to embryo transfer.
2. Users can extend or shorten the incubation time for in vitro transcription according to the amounts of final RNA products.
3. In cases where the concentration of mRNA is low, users can prepare two or three reaction tubes for the in vitro transcription reaction, and repeat the cleaning step to concentrate the solution.
4. Three different methods are available to prepare fertilization medium, depending on whether in vitro fertilization will be carried out using fresh, frozen-thawed, or cold-temperature-transported spermatozoa.
5. The degree of fertility varies greatly depending on the spermatozoa used. Spermatozoa with high fertility levels can be observed moving in a vortex manner with high motility at the boundary of the incubation medium. Conversely, spermatozoa which display low motility and poor homogeneity tend to be associated with low fertility levels.
6. Be sure to carry out all operations, from sacrificing the female and removing her oviducts to introducing the COCs into a drop of fertilization medium, in the shortest possible time (within 30 s). Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.
7. At this stage it is important that you identify and remove any parthenogenetic oocytes. If you do not do this, on the next day when they develop to the two-cell stage, it will be impossible to distinguish the fertilized oocytes from the parthenogenetic oocytes.
8. In the event that users prefer to perform microinjection at a later date, follow the protocol 3.5 to freeze the fertilized embryos.
9. Alternatively, electroporation can be utilized to introduce base editing tools into mouse embryos [12].

10. Users may be able to increase concentrations of BE or Target-AID mRNA and sgRNA.
11. It is recommended to inject at least 40–50 zygotes for each sgRNA to accurately evaluate the efficiency.
12. Targeted sequencing is usually the best way to confirm the introduction of desired mutations. Users may be able to perform PCR and subsequent digestion reactions to confirm the existence of desired mutations if any restriction site is introduced or removed by the introduced mutations.
13. Although the frequency is lower compared to original CRISPR/Cas9, double-strand DNA breaks and subsequent indel formation can still occur, which makes it difficult to determine the presence of desired point mutations. In addition, indel formation can occur on the same allele with the point mutations. TA cloning can be used to confirm the presence of desired mutations.
14. Embryo transfer into the oviduct is carried out by cutting the oviduct, inserting a capillary therein and expelling embryos toward the ampulla. Unfortunately, the oviducts of mice are small and the ducts are folded in a complicated manner. This makes it very difficult to insert the capillary into the oviduct towards the ampulla given that the insertion is made from above. To make this procedure easier, position the oviduct by changing the orientation of the Serrefine clamp and the mouse before starting the operation.
15. Because the folds in the oviduct vary between each mouse, look closely and adjust the position of the oviduct to make it easier to work on. If you are left-handed, position the oviduct so that you can easily carry out the procedure with your left hand.
16. When the glass capillary is first inserted into the drop, some liquid paraffin will remain on the outer surface of the drop as shown below. The embryos should be drawn into the glass capillary from the opposite side of the drop to avoid introducing any liquid paraffin. Evidence suggests that liquid paraffin which passes into the oviduct may have adverse effects on the development of the embryos into offspring.
17. If performed successfully, you should be able to see air bubbles through the wall of the ampulla. If you cannot expel the embryos and the air bubbles into the oviduct, pull the capillary back out just a little from the slit and expel the embryos again.
18. Transfer the embryos after adjusting the position and direction of the oviduct. If the oviduct is aligned parallel to the capillary, then it will be easier to insert the capillary into the oviduct.
19. It has been reported that base editing tools can induce sgRNA-independent off-target mutations due to the overexpression of

cytidine deaminase [13]. It is recommended to backcross mouse founders to inbred control wild-type animals for five generations to eliminate these unpredicted off-target mutations. Whole-genome sequencing should be considered to identify these sgRNA-independent off-target mutations.

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Chapter 10

Genome Editing in Mouse and Rat by Electroporation

Takehito Kaneko

Abstract

Many genome-edited mouse and rat strains have been produced using engineered endonucleases, including zinc finger nuclease (ZFN), *transcription activator-like effector nuclease* (*TALEN*), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. Especially, CRISPR-Cas9 is powerful tool that can be easy, rapid, and high-efficiency-produced new genome-edited strains. Furthermore, new technique, Technique for Animal Knockout system by Electroporation (TAKE), efficiently accelerate production of new strains by direct nuclease introduction into intact embryos using electroporation. This chapter presents a latest technical information in the production of genome-edited mouse and rat by TAKE method.

Key words Mouse, Rat, CRISPR/Cas9, In vitro fertilization, Electroporation, Fluorescent, Intact embryos, Embryo transfer

1 Introduction

Genome editing, zinc finger nuclease (ZFN), *transcription activator-like effector nuclease* (*TALEN*), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 are powerful tools for the production of new genetically engineered animal strains [1–6]. Microinjection method [7], which directly introduce endonucleases into pronuclear stage embryos, is the gold standard to produce new genome-edited strains (Chap. 17). This technique requires a high skill level to minimize cell damage during injection. We developed new nuclease introduction system into embryos by electroporation to accelerate the production efficiency of animals. Technique for Animal Knockout system by Electroporation (TAKE) can be introduced nucleases into intact embryos without weakening the zona pellucida by using a three-step electrical pulse system [8, 9]. TAKE method is possible to produce genome-edited animals without specialist skills, such as conventional microinjection method. At present, TAKE method has been modified and applicable to gene editing in various species.

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Table 1
Components of HTF and mKRB media

Components	HTF mg/100 mL (mM)	mKRB mg/100 mL (mM)
NaCl	594 (101.6)	553 (94.6)
KCl	35 (4.7)	36 (4.8)
$CaCl_2$	23 (2.0)	19 (1.7)
$MgSO_4$. $7H_2O$	5 (0.2)	29.3 (1.2)
KH_2PO_4	5 (0.4)	16 (1.2)
$NaHCO_3$	210 (25.0)	211 (25.1)
Na-lactate (60% syrup)	0.34 mL	0.19 mL
Na-pyruvate	4 (0.3)	6 (0.5)
D-glucose	50 (2.8)	100 (5.6)
Penicillin G	7	7
Streptomycin	5	5
Bovine serum albumin (BSA)	400	400

2 Materials

2.1 Preparation of Mouse and Rat Pronuclear Stage Embryos by In Vitro Fertilization (IVF)

1. Mature male and mature or immature female mice or rats.
2. Human tubal fluid (HTF) for mouse and rat in vitro fertilization (IVF) and embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C (*see* **Note 1**).
3. Sterile mineral oil.
4. 30–60 mm plastic culture dish.
5. CO_2 incubator.
6. Pregnant mare serum gonadotropin (PMSG).
7. Human chorionic gonadotropin (hCG).
8. Stereomicroscope.
9. Syringe (1 mL) with 30G steel needle.
10. Pair of small scissors.
11. Fine-tipped forceps.
12. Glass capillary pipettes.

2.2 Preparation of Mouse and Rat Pronuclear Stage Embryos by Natural Mating

1. Mature male and female mice or rats.
2. Human tubal fluid (HTF) for mouse embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C (*see* **Note 1**).
3. Modified Krebs-Ringer bicarbonate (mKRB) medium for rat embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C (*see* **Note 1**).
4. Sterile mineral oil.
5. 30–60 mm plastic culture dish.
6. CO_2 incubator.
7. Pregnant mare serum gonadotropin (PMSG).
8. Human chorionic gonadotropin (hCG).
9. Stereomicroscope.
10. Syringe (1 mL) with 30G steel needle.
11. A pair of small scissors.
12. Fine-tipped forceps.
13. Glass capillary pipettes.

2.3 Electroporation of Nucleases Into Intact Pronuclear Stage Embryos (TAKE Method)

1. Electroporator (NEPA 21: NEPA GENE Co. Ltd., Chiba, Japan) (Fig. 1a) (*see* **Note 2**).
2. Glass slide electrodes (1 mm gap; CUY501P1-1.5: NEPA GENE Co. Ltd.) (Fig. 1b) (*see* **Note 3**).
3. Cas9 protein.
4. tracrRNA.
5. crRNA for the target.
6. Single-strand oligo donor (ssODN).
7. Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA).
8. Stereomicroscope.
9. Glass capillary pipettes.

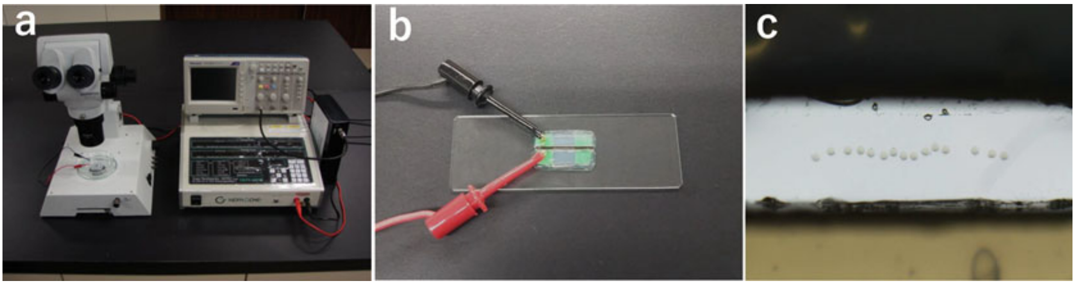

Fig. 1 (**a**) Electroporator NEPA 21. (**b**) 1 mm gap glass slide electrodes (CUY501P1-1.5). (**c**) Embryos are introduced between the electrodes filled with nuclease solution. (The part of figure is reproduced from Ref. [10])

2.4 Confirmation and Quantity Measurement of Nucleases in the Embryos after TAKE Method

1. Electroporator (NEPA 21: NEPA GENE Co. Ltd., Chiba, Japan).
2. Glass slide electrodes (1 mm gap; CUY501P1-1.5: NEPA GENE Co. Ltd.).
3. Cas9 protein.
4. ATTO550 labeled tracrRNA: tracrRNA-ATTO550 (#1075927, Integrated DNA Technologies, Inc. Coralville, IA, USA).
5. crRNA for the target.
6. Single-strand oligo donor (ssODN).
7. Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA).
8. Stereomicroscope.
9. Glass capillary pipettes.
10. Fluorescent inverted microscope.
11. ImageJ (https://imagej.nih.gov/ij/).

2.5 Embryo Transfer

1. Mature female mice or rats.
2. Vasectomized male mice or rats.
3. Isoflurane for anesthesia: 1%, 0.8 L/min for mouse, 2%, 1 L/min for rat.
4. Stereomicroscope.
5. A pair of small scissors.
6. Fine-tipped forceps.
7. Glass capillary pipettes.
8. 30G steel needle.
9. Wound clips.

2.6 Genotyping of Delivered Pups

1. Pair of small scissors.
2. FTA cards.
3. DNA extraction kit.
4. PCR system.
5. Electrophoresis system.
6. DNA sequencing system.

3 Methods

3.1 Preparation of Mouse and Rat Pronuclear Stage Embryos by IVF

1. Prepare two culture dishes with 200 μl drops of HTF medium covered with sterile mineral oil.
2. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.

3. Euthanize a male by CO_2 overdose and cervical dislocation.
4. Remove the cauda epididymides using a pair of small scissors.
5. Squeeze the sperm mass out of the epididymides using fine-tipped forceps.
6. Place the sperm mass in drops of HTF medium (*see* **Note 4**).
7. Capacitate sperm for 60 min at 37 °C under 5% CO_2 and 95% air.
8. Induce superovulation in females by intraperitoneal injection of PMSG (5–7.5 IU/body for mouse, 150–300 IU/kg for rat), followed by injection of hCG (5–7.5 IU/body for mouse, 75–300 IU/kg for rat) 48 h later.
9. Euthanize the females by CO_2 overdose and cervical dislocation, 13–15 h after hCG injection.
10. Remove the oviducts using a pair of small scissors (*see* **Note 5**).
11. Place the oviducts into mineral oil in another culture dish.
12. Collect the cumulus-oocyte complexes by puncturing the ampulla of oviducts using a steel needle.
13. Transfer the cumulus-oocyte complexes to drops of HTF medium.
14. Add the capacitated sperm suspension into the drops of HTF medium with the oocytes. The final sperm concentration is approximately 1×10^5 cells/mL for mouse and 5×10^5 cells/mL for rat.
15. Place the culture dish at 37 °C under 5% CO_2 and 95% air.
16. Collect the embryos that appear pronuclear 5 h after insemination using glass capillary pipettes.
17. Place the pronuclear stage embryos in a new drop of HTF medium at 37 °C under 5% CO_2 and 95% air until used for electroporation.

3.2 Preparation of Mouse and Rat Pronuclear Stage Embryos by Natural Mating

1. Use HTF medium for mouse or mKRB medium for rat to manipulate embryos.
2. Induce superovulation in females by intraperitoneal injection of PMSG (5–7.5 IU/body for mouse, 150–300 IU/kg for rat), followed by injection of hCG (5–7.5 IU/body for mouse, 75–300 IU/kg for rat) 48 h later.
3. After hCG injection, mate females with males overnight.
4. Confirm the presence of vaginal plugs to ensure mating has occurred.
5. Prepare four 50 μL drops of HTF or mKRB medium in the culture dish.
6. Cover drops with mineral oil in the culture dish.

7. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.
8. Euthanize females with CO_2 overdose and cervical dislocation.
9. Remove the oviducts using a pair of small scissors (*see* **Note 5**).
10. Flush ampulla with HTF or mKRB medium using a 1 mL syringe with a 30G steel needle.
11. Collect pronuclear stage embryos using glass capillary pipettes, and transfer to one of the four drops of medium (*see* **Note 6**).
12. Remove cumulus cells and other debris, and transfer the embryos to another drop of medium.
13. Place the pronuclear stage embryos in the fourth drops of medium and place at 37 °C under 5% CO_2 and 95% air until used for electroporation.

3.3 Electroporation of Nucleases Into Intact Pronuclear Stage Embryos (TAKE Method)

1. The electroporation procedure is the same for mouse and rat embryos (*see* **Note 7**).
2. Prepare 10 μL of nuclease solution at 100 μg/mL for Cas9 protein and 500 μg/mL of dualRNA (equal molar mixture of crRNA and tracrRNA) in Opti-MEM medium.
3. Add 100 μg/mL of ssODN in the nuclease solution when produce knock-in strain.
4. Introduce 10 μL of nuclease solution into the electrode (Fig. 1b).
5. Place 10–20 pronuclear stage embryos in a line between electrodes (Fig. 1c).
6. Connect the electrodes to the electroporator (Fig. 1a).
7. Set up the poring and transfer pulses (Table 2) on the electroporator.
8. Electroporate the intact embryos (*see* **Note 8**).
9. Place electroporated embryos into fresh drops of HTF or mKRB medium at 37 °C under 5% CO_2 and 95% air until embryo transfer.

Table 2
Recommended pulse setting using 1 mm gap glass slide electrodes (CUY501P1-1.5)

	Voltage (V)	Pulse width (ms)	Pulse interval (ms)	Number of pulses	Decay rate (%)	Polarity
Poring pulse	40	3.5	50	4	10	+
Transfer pulse	15	50	50	5	40	+/−

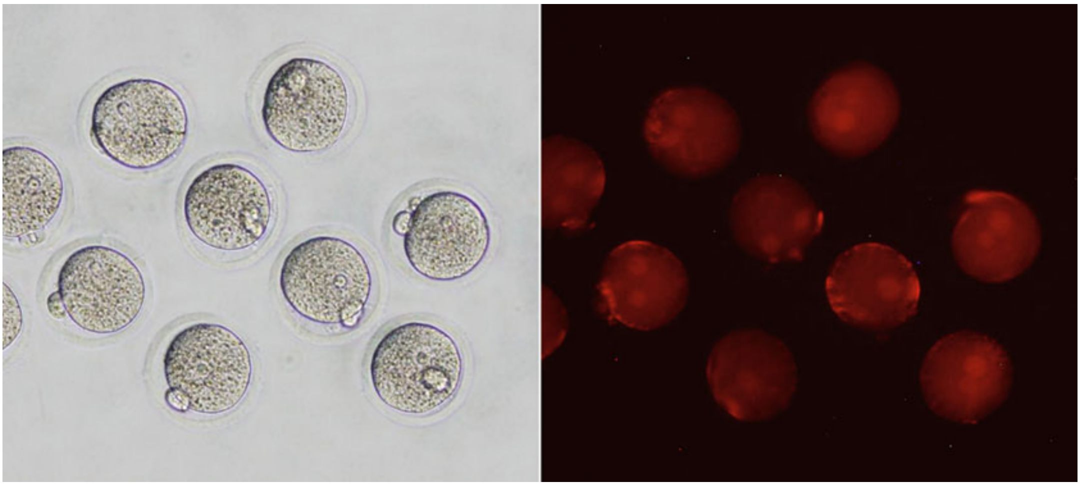

Fig. 2 Fluorescence of embryos introduced Cas9 protein and fluorescent dualRNA (tracrRNA-ATTO550 and crRNA) by TAKE method. (The part of figure is reproduced from Ref. [11])

3.4 Confirmation and Quantity Measurement of Nucleases in the Embryos After TAKE Method

1. Use tracrRNA-ATTO550 for confirmation and quantity of nucleases in the embryos after TAKE method [11].
2. Prepare 10 μL of nuclease solution at 100 μg/mL for Cas9 protein and 500 μg/mL of dualRNA (equal molar mixture of crRNA and tracrRNA-ATTO550) in Opti-MEM medium.
3. Introduce 10 μL of nuclease solution into the electrode (Fig. 1b).
4. Place 10–20 pronuclear stage embryos in a line between electrodes (Fig. 1c).
5. Connect the electrodes to the electroporator (Fig. 1a).
6. Set up the poring and transfer pulses (Table 2) on the electroporator.
7. Electroporate the intact embryos (*see* **Note 7**).
8. Confirm fluorescence in the embryos using fluorescent-inverted microscope (Fig. 2).
9. Measure quantity of nucleases by fluorescent intensity using ImageJ.

3.5 Embryo Transfer

1. Perform the same embryo transfer procedure in mouse and rat.
2. Mate female mice or rats with vasectomized males on the day before transfer.
3. Confirm the presence of vaginal plugs of females to ensure mating has occurred (*see* **Note 9**).
4. Anesthetize a female using isoflurane.
5. Expose the ovary, oviduct, and part of the uterus through an abdominal incision.

6. Make a small hole in the upper ampulla using a 30G steel needle.
7. Aspirate 5–10 embryos into a glass capillary pipette with a few small air bubbles (*see* **Note 10**).
8. Insert this capillary pipette into the hole in the oviduct.
9. Transfer the embryos with air bubbles.
10. Return the ovary, oviduct, and uterus back inside the body cavity, and seal the incision with wound clips.
11. Transfer more embryos into the other oviduct.
12. Deliver pups at gestation day 19 for mouse or day 21 for rat.

3.6 Genotyping of Delivered Pups

1. Collect blood from 3-week-old pups.
2. Blot blood samples onto the FTA cards.
3. Extract genomic DNA from blood samples using a DNA extraction kit.
4. Amplify the target sequences by PCR.
5. Electrophorese the PCR products on agarose gels.
6. Sequence the PCR products to confirm the mutations (*see* **Note 11**).

4 Notes

1. All media can be stored for up to 1 month.
2. The device of other companies can be used, although electrical conditions were different.
3. Five mm gap glass slide electrodes (CUY520P5: NEPA GENE Co. Ltd.) is easy to manipulate embryos.
4. Handle gently to maintain high sperm motility.
5. Collect oviducts immediately after euthanasia to avoid damage to oocytes.
6. If cumulus cells are attached to embryos, remove using 0.1% hyaluronidase.
7. Embryo derived from freeze-dried and frozen sperm and frozen embryo can be used for TAKE method [12–15].
8. TAKE method can use intact embryos without any chemical treatment, such as zona-thinning using Tyrode's acid solution.
9. Pseudopregnancy can be introduced using sonic vibration instead of vasectomized male [16].
10. Embryo transfer at the two-cell stage is suitable for subsequent embryo development.

Table 3
Development of mouse and rat embryos introduced nucleases by TAKE method

Animals	No. of embryos examined	No. of embryos survived (%)	No. of two-cell embryos (%)	No. of offspring (%)	No. of knockout offspring (%)
Mouse	29	29 (100)	26 (90)	11 (42)	10 (91)
Rat	25	25 (100)	25 (100)	17 (68)	17 (100)

The data are reproduced from Refs. [10, 17]

11. Typical results in production of knockout strains are shown in Tables 3.

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Chapter 11

Generation of Floxed Mice by Sequential Electroporation

Takuro Horii, Ryosuke Kobayashi, and Izuho Hatada

Abstract

Generation of conditional knockout mice using the Cre-loxP system is essential for the analysis of gene functions. The use of CRISPR-Cas9 in combination with two sets of guide RNAs and single-stranded oligonucleotides including loxP sites enables simultaneous insertion of two loxP sequences. Unfortunately, this method induces double-strand breaks at two sites in the same chromosome, which causes an undesirable large chromosomal deletion and reduces the flanked loxP (flox) rate. To overcome this problem, we have developed a method that sequentially introduces each loxP sequence by electroporation at the one- and two-cell embryonic stages, respectively. This sequential electroporation method improves the floxing efficiency compared with the conventional simultaneous method, leading to a high yield of offspring with floxed alleles.

Key words Cre-loxP, CRISPR-Cas9, Electroporation, Flox, Knock-in, loxP, Mouse

1 Introduction

Various genome engineering technologies have been established to produce gene knockout mice, but a significant number result in embryonic lethal phenotypes [1]. A conditional knockout system allows the control of spatial and temporal genetic modifications, which can overcome this problem. The most commonly used conditional knockout system, the Cre-loxP system, utilizes a site-specific Cre recombinase and the loxP site consisting of a unique 34 bp sequence [2]. Cre-mediated recombination occurs by deletion or inversion of a genetic region of interest, flanked by two loxP sites (floxed), leading to targeted gene modification in Cre-expressing cells.

Until a decade ago, the generation of floxed mice was a time-consuming and complex process that involved homologous recombination in embryonic stem cells, microinjection of these cells to produce chimeric mice, and crossbreeding of chimeric mice with wild-type mice to produce heterozygous offspring. However, advances in genome editing technology, such as clustered regularly

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interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) [3], enable the generation of knockout mice by delivery of Cas9 nuclease and a guide RNA (gRNA) to target complementary DNA sequences. In addition, DNA insertions and precise point mutations can be induced when this technique is used in combination with a single- or double-stranded donor DNA template homologous to the target sequence flanking the double-strand break (DSB) site [4–6]. Notably, floxed mice can be generated by simultaneous injection of Cas9 and two pairs of gRNAs and single-stranded oligodeoxynucleotides (ssODNs) containing loxP sites into mouse zygotes [6–9]. This powerful technique eliminates the need to construct knock-in (KI) vectors and allows floxed mice to be obtained in a short amount of time. However, the method using two pairs of gRNAs and ssODNs induces two DSBs in the same chromosome, resulting in undesirable chromosomal deletions and reduced floxed rates (Fig. 1a) [10, 11].

To overcome this problem, we investigated a method that sequentially introduces each loxP site by electroporation at the one- and two-cell embryonic stages, respectively (Fig. 1b)

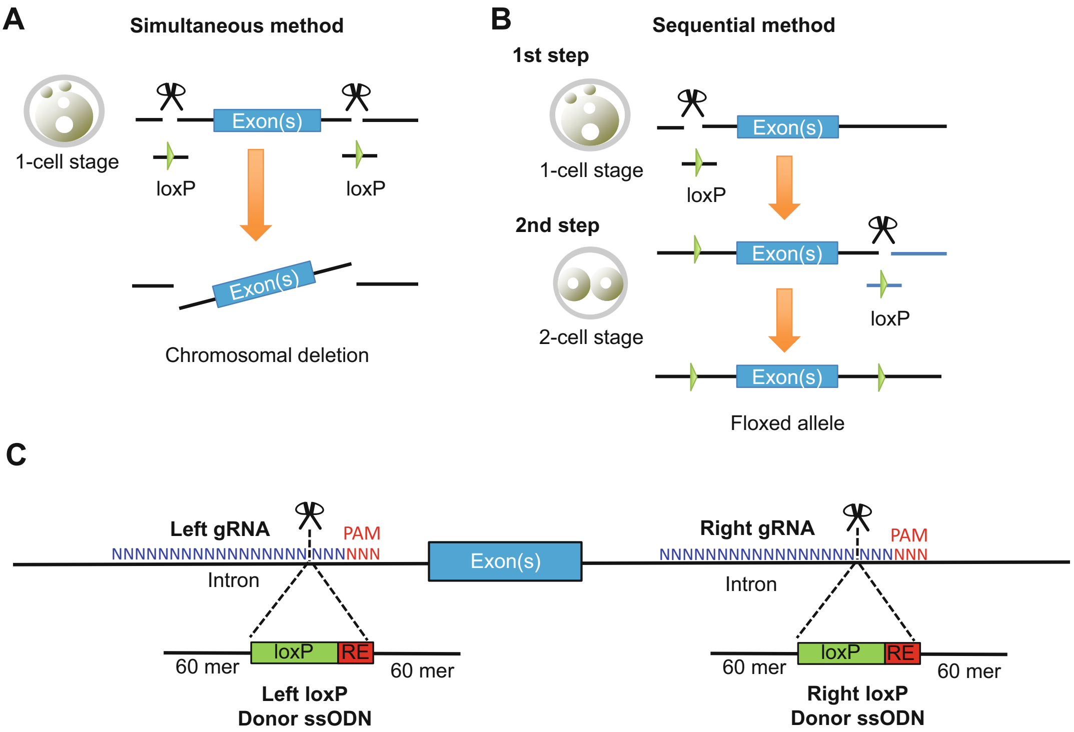

Fig. 1 Schematic of experimental procedures for (**a**) an ordinary simultaneous method and (**b**) a novel sequential method to generate floxed mice. (**c**) Donor loxP ssODNs with 60 bp homology to sequences on each side of a gRNA-mediated DSB site. To facilitate the detection of correct loxP insertions, ssODNs were engineered to contain a restriction enzyme site (RE)

[10, 12]. Sequential electroporation improves the floxing efficiency compared with conventional simultaneous microinjection or electroporation, resulting in higher yields of progeny with floxed alleles. This chapter serves as an instructional guide for the generation of floxed mice by sequential electroporation of CRISPR-Cas9 gRNAs and ssODNs.

2 Materials

2.1 Embryo Collection and Culture

1. Mice such as B6D2F1 mice, 8–10 weeks of age (female) and >8 weeks of age (male).
2. Pregnant mare's serum gonadotropin (PMSG).
3. Human chorionic gonadotropin (hCG).
4. M16 embryo culture medium.
5. M2 embryo culture medium.
6. M2-hyaluronidase medium: M2 medium containing hyaluronidase (300 U/mL).
7. Phosphate-buffered saline (PBS) containing 0.1% polyvinylpyrrolidone (PVP).
8. Paraffin oil.
9. 60 mm plastic dish.

2.2 Genome Editing

1. UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGateway).
2. CRISPR design tools such as CRISPRdirect (http://crispr.dbcls.jp/) and CRISPOR (http://crispor.tefor.net/).
3. gRNAs: both single gRNA (sgRNA) and CRISPR RNA (crRNA): Trans-activating crRNA (tracrRNA) are available for production of floxed mice (*see* **Note 1**).
4. Donor loxP ssODNs (*see* **Note 2**).
5. RNase-free duplex buffer: 30 mM HEPES, pH 7.5 containing 100 mM potassium acetate.
6. Cas9 nuclease protein.
7. Opti-MEM I medium.
8. Electrode (1 mm gap and 10 mm length) such as LF501PT1–10 (BEX, Tokyo, Japan).
9. Electroporator such as CUY21EDIT (BEX).
10. Binocular stereo microscope.

2.3 Embryo Transfer

1. Pseudopregnant foster mice such as ICR mice, >8 weeks of age.
2. Vasectomized mice such as ICR mice, >8 weeks of age.

2.4 Assay for Genome Modification

1. PCR reagent.
2. DirectPCR Lysis Reagent (102-T; Viagen Biotech, Los Angeles, CA).
3. Proteinase K solution.
4. Primer set flanking the target sequences of gRNAs (*see* **Note 3**).
5. Primer set flanking the target exon(s) and two loxP sites.
6. T7 endonuclease I such as M0302S (NEB, Ipswich, MA).
7. 0.25 M EDTA.
8. Restriction enzyme.
9. TA cloning vector such as a TOPO TA Cloning Kit (Thermo Fisher Scientific, Waltham, MA).
10. Equipment for gel electrophoresis or capillary and microchip electrophoresis such as MCE–202 (MultiNA; Shimadzu, Kyoto, Japan).

3 Methods

3.1 Embryo Collection and Culture

1. Induce superovulation of female B6D2F1 mice (*see* **Note 4**) by administering 7.5 U of PMSG followed by 7.5 U of hCG 48 h later (*see* **Note 5**). After administration of hCG, mate females overnight with males. The following morning, check for the presence of a vaginal plug to determine whether copulation was successful (*see* **Note 6**).
2. On the morning of embryo collection, place several flat drops of M16 medium (25 μL/drop) on a 60 mm dish, and cover them with paraffin oil. Equilibrate M16 medium at 37 °C in air containing 5% CO_2.
3. At 21 h after hCG administration, sacrifice female mice and dissect the oviducts.
4. Transfer the oviducts to a 35 mm Petri dish containing 1.5 mL of M2 medium at room temperature, and then remove the blood by shaking the dish.
5. Transfer all oviducts to a new 35 mm Petri dish containing 1.5 mL of M2-hyaluronidase medium.
6. Cut the oviduct ampulla using a 27-gauge hypodermic needle attached to a 1 mL syringe, and gently squeeze out the cumulus–oocyte complex into M2-hyaluronidase medium.
7. Incubate at 37 °C for several minutes until the cumulus cells detach.
8. Collect the fertilized eggs using an embryo transfer pipette, and transfer to a new dish containing M2 medium. Wash the embryos three times, and then culture in M16 medium at 37 °C in air containing 5% CO_2 until electroporation.

3.2 gRNA Selection

1. Obtain the target DNA sequence from a genome database such as the UCSC Genome Browser.
2. Select the gRNA with the highest cleavage activity from the left and right gRNAs using mouse embryos at the blastocyst stage. Design and prepare at least three gRNAs targeting each intron flanking the target exon(s) using the CRISPR design tools (Fig. 1c).
3. If using crRNA as gRNA, combine equal volumes of crRNA and tracrRNA in duplex buffer in a PCR tube, heat in a thermal cycler at 95 °C for 5 min, and then let the tube cool slowly to room temperature (*see* **Note 7**).

Component (stock concentration)	Amount (final concentration)
crRNA (100 μM)	1.5 μL (18 μM)
tracrRNA (100 μM)	1.5 μL (18 μM)
RNase-free duplex buffer	5.3 μL
Total volume	**8.3 μL**

4. Perform electroporation using one-cell stage embryos at 24–26 h after hCG administration. Set up the electroporation system as shown in Fig. 2a, b.
5. Immediately prior to electroporation, prepare the genome editing mixture including 100 ng/μL Cas9 protein and 3 μM crRNA:tracrRNA (or sgRNA) in Opti-MEM I buffer as shown below.

Component (stock concentration)	Amount (final concentration)
Cas9 nuclease protein (1 μg/μL)	1 μL (100 ng/μL)
crRNA:tracrRNA (18 μM)	1.7 μL (3 μM = 100 ng/μL)
Opti-MEM I	7.3 μL
Total volume	**10 μL**

6. Incubate the mixture at room temperature for 10 min, and then store on ice until use (*see* **Note 8**).
7. Place several 50 μL drops of Opti-MEM I on the lid of a 60 mm dish.
8. Using an embryo transfer pipette, transfer about 30 embryos to an Opti-MEM I drop. Wash the embryos twice in Opti-MEM I buffer.
9. Turn on the electroporator, and set the pulse settings to 30 V, 3 ms ON +97 ms OFF, seven electric pulses.

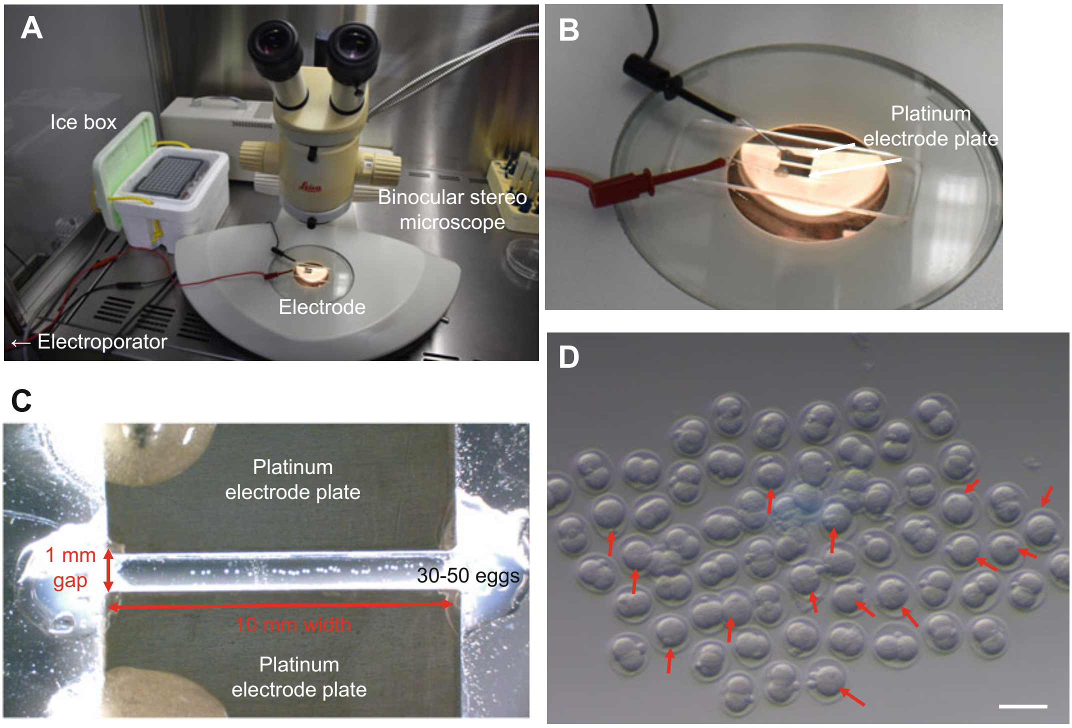

Fig. 2 Sequential electroporation system. (**a**, **b**) Electroporation system used in our laboratory. (**c**) The electrode immediately before electroporation. (**d**) Electroporation at the two-cell stage frequently induces blastomere fusion. Arrows indicate fused embryos after the second step electroporation. Scale bar, 0.1 mm

10. Fill the electrode gap with 5 μL of Opti-MEM I buffer.
11. Apply electrical charge to the electrode in order to check for energization. Once energized, air bubbles can be seen forming in the gap between the electrodes (*see* **Note 9**).
12. Replace Opti-MEM I buffer with 5 μL of the genome editing mixture including Cas9 and gRNA.
13. Place about 30 embryos in a line in the electrode gap (Fig. 2c).
14. Start electroporation.
15. When electroporation is completed, put the embryos back into an Opti-MEM I drop, wash three times with Opti-MEM I, and then put back into M16 medium.
16. Wash the electrode three times with 5 μL of Opti-MEM I, and then fill the gap with the next gRNA mixture.
17. After all electroporation is completed, incubate embryos at 37 °C for an additional 30 min.
18. Separate surviving embryos and culture in M16 medium at 37 °C in air containing 5% CO_2 for 4 days until they reach the blastocyst stage.

19. Wash blastocysts twice with PBS containing 0.1% PVP.
20. Transfer more than 15 embryos to 15 μL of DirectPCR Lysis Reagent containing proteinase K (100 μg/mL) in a PCR tube.
21. Incubate at 55 °C for 5 h and then at 95 °C for 10 min using a thermal cycler.
22. Perform PCR amplification using the primer sets flanking the gRNA target sequences in a total volume of 20 μL using 2 μL of template DNA.
23. Make the following mixture using the 100 ng of PCR product.

Component	**Amount**
PCR product (100 ng)	X μL
10× NEBuffer 2	1 μL
Distilled water	8.5-X μL
Total volume	**9.5 μL**

24. Anneal the PCR products in a thermal cycler using the following conditions:

Step	**Temperature**	**Ramp rate**	**Time**
Initial denaturation	95 °C		5 min
Annealing	95–85 °C 85–25 °C	−2 °C/s −0.1 °C/s	
Hold	4 °C		Hold

25. Add 0.5 μL of T7 endonuclease I to the annealed PCR products and incubate for 15 min at 37 °C.
26. Stop the reaction by adding 0.75 μL of 0.25 M EDTA.
27. Analyze the percentage of cleaved DNA among total DNA by capillary and microchip electrophoresis (Fig. 3). Three technical replicates should be performed for each gRNA sample. Select the gRNA with the highest DNA cleavage activity among the three candidate gRNAs in each intron.

3.3 Donor loxP ssODNs

Design and prepare loxP ssODNs with 60 bp homology to sequences on each side of each gRNA-mediated DSB site (Fig. 1c). To facilitate the detection of correct loxP insertions, each ssODN is engineered to contain a restriction enzyme site in addition to the loxP sequence. It is important to ensure that the ssODN does not contain GGGG sequences because this will reduce its yield.

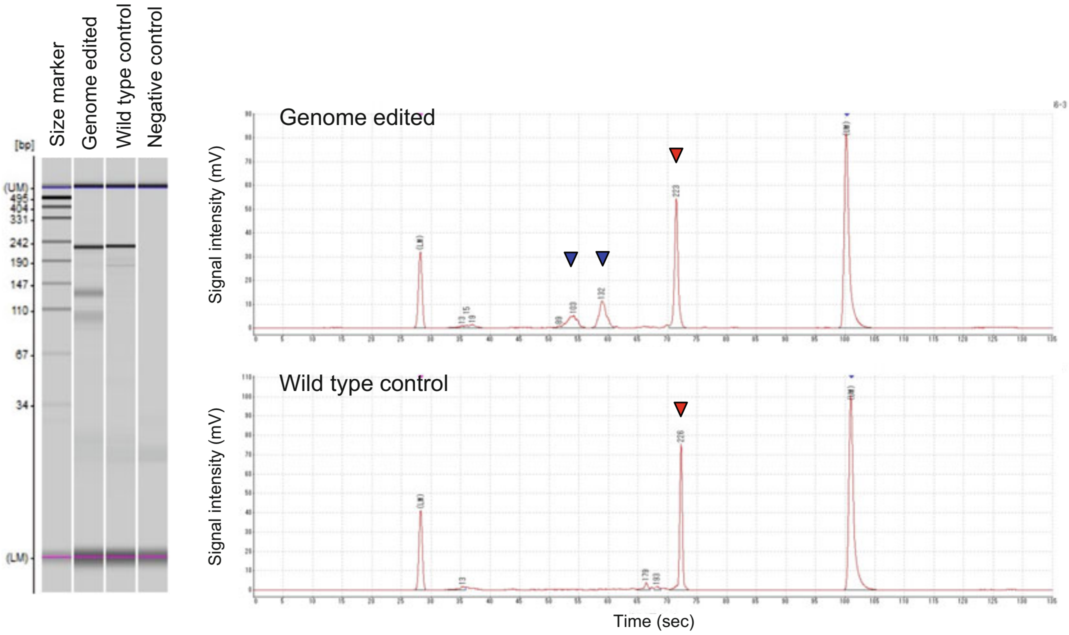

Fig. 3 gRNA selection by the T7 endonuclease I assay. Data are analyzed by capillary and microchip electrophoresis (MCE-202 MultiNA). Red arrowheads indicate undigested PCR product and blue arrowheads indicate digested PCR product. DNA cleavage activity is calculated as the percentage of cleaved DNA (blue arrowheads) among total DNA (blue and red arrowheads)

As an example, a donor loxP ssODN sequence for a *Tet3* intron [10] is shown below:

actgatctgagggttatctctgtggaagggcaggagcaggccatctaagccaacactaccAT
AACTTCGTATAATGTATGCTATACGAAGTTAT (loxP)
gaattc(*Eco*RI)
tagggcttcaagaatccactctacttccctcctcacaagtagcaaaacccattagttggc

3.4 The First Step of Electroporation

1. Perform the first step of electroporation using one-cell stage embryos at 24–26 h after hCG administration. Set up the electroporation system as shown in Fig. 2a, b.
2. Immediately prior to electroporation, prepare the genome editing mixture including 100 ng/μL Cas9 protein, 3 μM crRNA:tracrRNA (or sgRNA), and 400 ng/μL ssODN in Opti-MEM I buffer as shown below.

Component (stock concentration)	Amount (final concentration)
Cas9 nuclease protein (1 μg/μL)	1 μL (100 ng/μL)
crRNA:tracrRNA (18 μM)	1.7 μL (3 μM = 100 ng/μL)
ssODN (100 μM)	0.76 μL (7.6 μM = 400 ng/μL)

(continued)

Component (stock concentration)	Amount (final concentration)
Opti-MEM I	6.54 μL
Total volume	**10 μL**

3. Incubate the mixture at room temperature for 10, min and then store on ice until use (*see* **Note 8**).
4. Place several 50 μL drops of Opti-MEM I on the lid of a 60 mm dish.
5. Using an embryo transfer pipette, transfer 30–50 embryos to an Opti-MEM I drop. Wash the embryos twice in Opti-MEM I buffer.
6. Turn on the electroporator, and set the pulse settings to 30 V, 3 ms ON +97 ms OFF, seven electric pulses.
7. Fill the electrode gap with 5 μL of Opti-MEM I buffer.
8. Apply electrical charge to the electrode in order to check for energization. Once energized, air bubbles can be seen forming in the gap between the electrodes (*see* **Note 9**).
9. Replace Opti-MEM I buffer with 5 μL of the genome editing mixture including Cas9, gRNA, and ssODN.
10. Place 30–50 embryos in a line in the electrode gap (Fig. 2c).
11. Start electroporation.
12. When electroporation is completed, put the embryos back into an Opti-MEM I drop, wash three times with Opti-MEM I, and then put back into M16 medium.
13. Similarly, transfer the second set of embryos into the genome editing mixture on the electroporation chamber (*see* **Note 10**).
14. After all electroporation is completed, incubate embryos at 37 ° C for an additional 30 min.
15. Separate surviving embryos, and culture in M16 medium at 37 °C in air containing 5% CO_2 until the next day.

3.5 The Second Step of Electroporation

1. Perform the second step of electroporation using two-cell stage embryos at 42–44 h after hCG administration. The second step of electroporation is performed in the same way as the first step of electroporation. However, it should be noted that electroporation at the two-cell stage frequently induces blastomere fusion (Fig. 2d).
2. Remove the fused embryos about 1 h after electroporation (*see* **Note 11**).
3. Culture embryos in M16 medium at 37 °C in air containing 5% CO_2 until embryo transfer.

3.6 Embryo Transfer

1. Transfer embryos from the two-cell stage to the morula stage into the ampulla of the oviduct (about 20 embryos per oviduct) of pseudopregnant foster females at 0.5 days post-coitum (dpc). Alternatively, embryos at the blastocyst stage can be transferred into uterine horns of pseudopregnant females at 2.5–3.5 dpc (*see* **Note 12**).
2. Pups will be born at 19.5 dpc (*see* **Note 13**).
3. Extract genomic DNA from the tail tips of mice.

3.7 Assay for LoxP Insertion

1. To detect insertion of loxP sites, perform PCR-RFLP analysis using primers flanking the targeted exon(s) and two loxP sites (Fig. 4a).
2. Digest PCR products with each and then both restriction enzymes that can cleave loxP-inserted alleles but not wild-type alleles.
3. Analyze PCR products by gel electrophoresis or capillary and microchip electrophoresis (Fig. 4b). If a loxP site is inserted into the target intron, the PCR product is digested by each

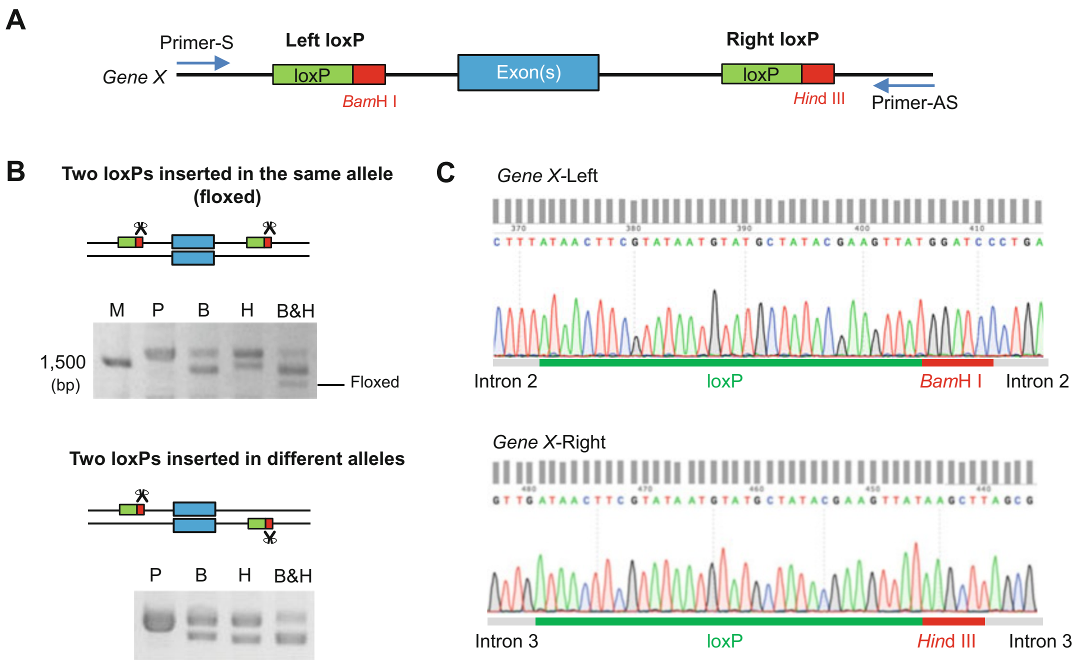
A
Primer-S
Left loxP
Gene X
loxP
BamH I
Exon(s)
Right loxP
loxP
Hind III
Primer-AS
B
Two loxPs inserted in the same allele (floxed)
M
P
B
H
B&H
1,500
(bp)
Floxed
Two loxPs inserted in different alleles
P
B
H
B&H
C
Gene X-Left
C T T T A T A A C T T C G T A T A A T G T A T G C T A T A C G A A G T T A T G G A T C C C T G A
Intron 2
loxP
BamH I
Intron 2
Gene X-Right
G T T G A T A A C T T C G T A T A A T G T A T G C T A T A C G A A G T T A T A A G C T T A G C G
Intron 3
loxP
Hind III
Intron 3

Fig. 4 Detection of floxed alleles. (**a**) Schematic of a representative floxed allele including two loxP sites flanking the targeted exon(s). (**b**) The first screening for floxed alleles by PCR-RFLP. If a loxP site is inserted into the target intron, the PCR product will be digested by each restriction enzyme. If both loxP sites are inserted into the same allele, a smaller PCR band will appear after double digestion. M marker, P PCR product before digestion, B *Bam*H I digestion to detect left loxP KI, H *Hin*d III digestion to detect right loxP KI, B&H *Bam*H I and *Hin*d III double digestion to detect the floxed allele. (**c**) Confirmation of loxP KI by TA cloning followed by Sanger sequencing

restriction enzyme. If both loxP sites are inserted into the same allele, a smaller PCR band will appear after double digestion.

4. Clone the PCR products derived from putative floxed alleles into a TA cloning vector and confirm the sequence of each allele (Fig. 4c) (*see* **Note 14**).

4 Notes

1. Designed sgRNAs or crRNAs and tracrRNAs are purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA, USA). We usually use Alt-R® CRISPR-Cas9 crRNA (2 nmol) and Alt-R® CRISPR-Cas9 tracrRNA (20 nmol). Resuspend each crRNA and tracrRNA to a concentration of 100 μM using TE (IDTE) and store at −80 °C.
2. PAGE-purified ssODNs are purchased from IDT (PAGE Ultramer® DNA Oligo). Resuspend to a concentration of 100 μM using TE (IDTE) and store at −80 °C.
3. Typically, design a primer set that amplifies a PCR product of about 200–300 bp.
4. Inbred strains such as C57BL/6 are also available [12], but we usually use hybrid B6D2F1 mice for flox production. The main advantage of using hybrid mice is the high survival rate of embryos both in vitro and in vivo.
5. In our laboratory, we inject ten females with PMSG and hCG at 3:00–4:00 PM (assuming a light period of 8:00 AM to 8: 00 PM).
6. Vaginal plugs should be checked in the early morning because they are expelled over time.
7. gRNA solution can be stored at −80 °C for up to 1 year. Do not repeatedly freeze and thaw because this promotes degradation of RNA. It is also important to use RNase-free TE or pure water to dilute the gRNA mixture. Do not use diethylpyrocarbonate-treated water, which is toxic to embryos.
8. This should be used up by the end of the day.
9. Under the same condition as ours (CUY21EDIT electroporator and LF501PT1–10 electrode), the current is 0.20 A. However, a current of 0.16–0.24 A is acceptable.
10. The genome editing mixture can be used up to two times. Keep the electrode on ice between operations to prevent evaporation.
11. These fused embryos cannot develop to term because they are tetraploidized [13].

12. It is better to transfer the manipulated embryos to oviducts as soon as possible after the second step electroporation. Alternatively, embryos can be cultured until the blastocyst stage to determine the developmental rate in vitro or genome editing efficiency.
13. When embryos from B6D2F1 mice are used, about 20% of transferred embryos are born as progeny.
14. More than two mutant alleles are identified in an individual mouse on some occasions. This is caused by mosaicism of somatic cells in mice generated by CRISPR-Cas9 injection. Therefore, sequence analyses of mutant alleles should be performed using multiple clones. In addition, the mutation profile of the germline in founder (F0) mice is sometimes different from that of tail tips used for genotyping; therefore, sequences of the mutant alleles should be checked again in F1 offspring.

Acknowledgments

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Chapter 12

Efficient Detection of Flox Mice Using In Vitro Cre Recombination

Ryosuke Kobayashi, Takuro Horii, and Izuho Hatada

Abstract

Advances in CRISPR/Cas9 genome editing technologies have allowed for the rapid generation of Cre-loxP conditional knockout mice. However, current strategies for genotyping flox mice, typically based on Sanger sequencing following cloning of target sequences from dozens of pups, are time-consuming. Here, we describe a rapid screening method for flox mice, using in vitro Cre recombination that can be performed using simple enzymatic reactions and enables detection of functional flox mouse within 1 day. In addition, we introduce an efficient strategy for subsequent sequence analysis by cloning of floxed regions using the In-Fusion system. Our genotyping pipeline reduces laborious tasks and thus contributes to the rapid selection of accurately edited flox mice.

Key words CRISPR/Cas9, Conditional knockout, Cre/loxP, Genotyping, Mouse

1 Introduction

The development of new genome editing technologies, especially clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISRP/Cas9), has facilitated the introduction of sequence-specific genetic mutations in a variety of organisms [1, 2]. The basic principle of genome editing is based on the repair process that occurs following double-strand breaks in genomic DNA induced by Cas9 nucleases. As the regulation of double-strand break repair is largely dependent on the host cell and the efficiency of precise repair is generally not high, unexpected mutagenesis often occurs following such editing events [3]. In addition, multiple editing alleles may be observed in cells and animals that have undergone genome editing. As a result, animals born following genome editing events in early embryogenesis frequently exhibit mosaicism in their genomes [4]. Therefore,

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validation of editing outcomes and selection of edited animals or cells that contain precisely edited alleles are essential steps for efficient production of genome-edited biomaterials.

The generation of genetically engineered mice is one area that has been greatly advanced by the development of genome editing technologies [5]. By applying genome editing to fertilized eggs, it is possible to achieve a significant reduction in the time and effort required to generate knockout and knock-in mice. Conditional knockout mice, which allow spatiotemporal control of gene knockout, are an important tool in recent biological science because they allow functional analysis of target genes in vivo, while avoiding the embryonic lethality caused by systemic knockout of essential genes [6]. Cre/loxP is the most commonly used system for generation of conditional knockouts, where Cre recombinase mediates deletion of a region of interest flanked by two loxP sequences (floxed) [7]. To introduce floxed alleles by genome editing, it is necessary to knock-in loxP sequences in *cis* (i.e., two loxP sequences are inserted into the same allele, rather than different alleles) at positions several kilobases apart [8–11]. The sequential method, in which genome editing is performed stepwise in early embryos, suppresses gene deletion caused by simultaneous genome editing and greatly increases the efficiency of flox mice generation (*see* Chap. 11 *for method of flox mice generation by sequential genome editing*) [12, 13]. Despite these technological advances, however, unintended consequences, such as mutations in the loxP sequence or knocking-in of two loxP sequences at different alleles (i.e., in *trans*), are unavoidable. Determination of sequences by Sanger sequencing following cloning of target regions is necessary to select mice that have the desired mutation. Given that the majority of pups that have undergone genome editing possess unintended genotypes, it is generally necessary to screen dozens of pups in order to identify a mouse with the desired genotype. Because it is very laborious to apply cloning and sequencing procedures to all born mice, screening for candidates is essential in order to minimize subsequent cloning tasks. One strategy for this is to co-insert restriction sites along with loxP to allow detection of insertion events or allelic information (i.e., *cis* or *trans*) by PCR-restriction fragment length polymorphism (PCR-RFLP) (Fig. 1). Although this screening strategy has economic benefits and only requires readily available laboratory equipment, the results from this method are based on indirect observation of knock-in events and do not provide proof of insertion of functional floxed alleles. Recently, a multiplex genotyping tool based on long-read sequencing technology was reported; however, this technique requires bioinformatic analysis and this seems to present a barrier to its use for many scientists [14]. It is therefore essential to develop a rapid and easy screening method for flox mice that will reduce the time and effort required for subsequent cloning and sequencing analysis.

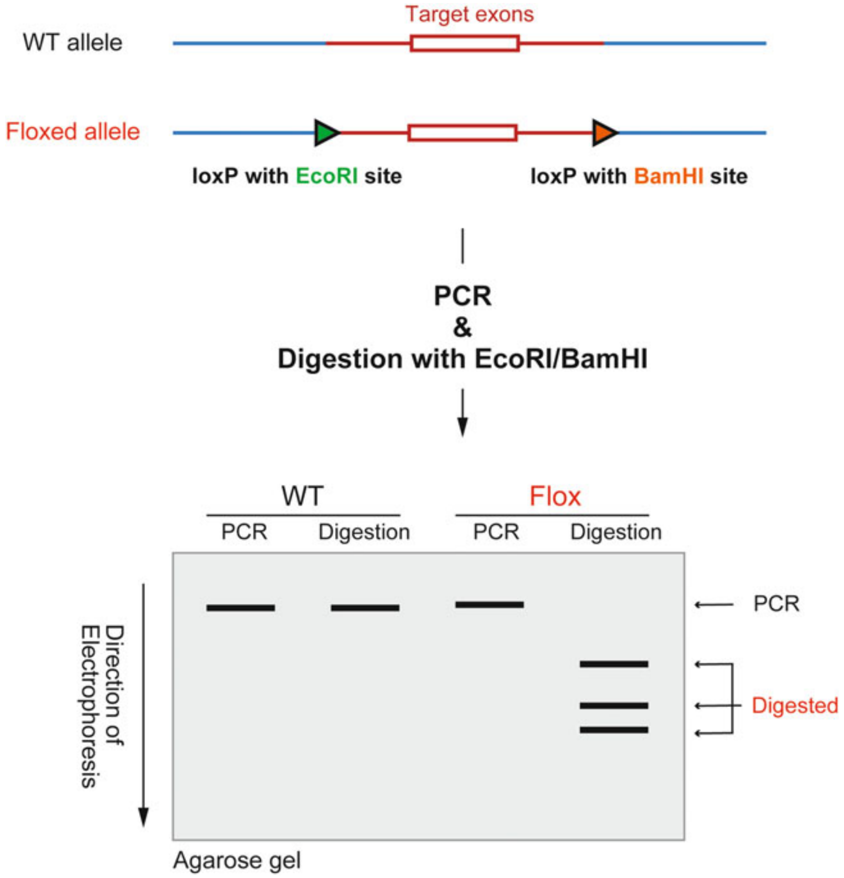

Fig. 1 Conventional screening of flox mice using PCR-RFLP. To aid genotyping, restriction sites (e.g., GAATTC for EcoRI, or GGATCC for BamHI) are often inserted along with loxP. PCR products for floxed region are digested by given restriction enzymes. If sample DNA contains floxed alleles, digested bands are observed by electrophoresis

Here, we introduce an efficient method for screening gene-edited mice for functional floxed alleles (a schematic of the entire process is illustrated in Fig. 2). In this method, which we have named "*in vitro* Cre recombination method," the full length of the floxed region is amplified by PCR (the first PCR), followed by a recombination reaction using Cre recombinase in a tube (i.e., in vitro). PCR products derived from functional floxed alleles are specifically recombined and shortened, while those derived from other alleles (wildtype or incomplete editing) are not changed. Finally, shortened fragments are detected by a second PCR, and animals with floxed alleles are identified. The entire process can be performed by enzymatic reaction alone, thus reducing the time and effort required to screen for gene edited flox mice with the desired insertion.

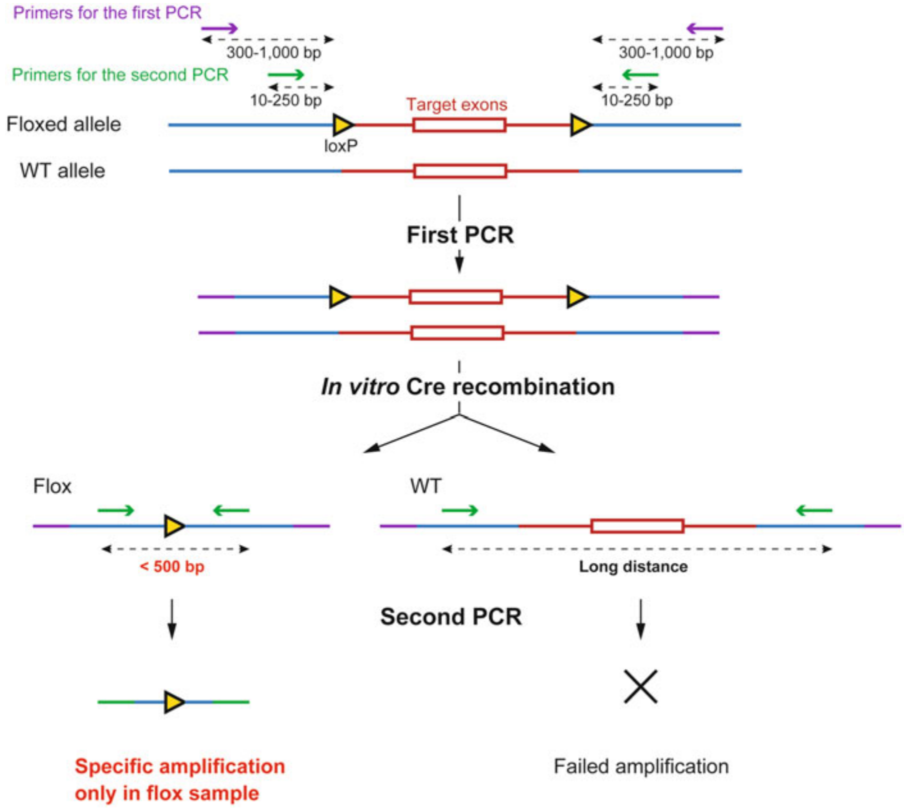

Fig. 2 Schematic showing detection of floxed alleles using in vitro Cre recombination

Although in vitro Cre recombination enables rapid detection of flox mice, it is still important to confirm the DNA sequence of the region around the loxP insertion site to avoid problems in later experiments. As described above, flox mice established by genome editing (even if their functionalities are proven by in vitro Cre recombination screening) often possess not only floxed alleles but also other alleles in a heterogeneous or mosaic manner. Thus, it is necessary to accurately isolate the floxed allele by cloning for subsequent sequence analysis. The most commonly used cloning method is based on 3′-A overhangs of PCR products (i.e., TA-cloning); however, cloning of the entire region of the floxed allele, which spans several kilobase pairs, is often inefficient in this system. Instead, we have been able to efficiently clone long floxed regions using the In-Fusion cloning system that is based on homology-mediated assembly of DNA fragments. In this system, a set of DNA fragments with homologous ends is seamlessly joined in a ligase-independent manner by a proprietary enzyme that possesses exonuclease activity (Fig. 3) [15]. The overlap homology region (>15 bp) can be engineered by inclusion in PCR primers. Thus, the advantage of In-Fusion cloning is not only the relatively

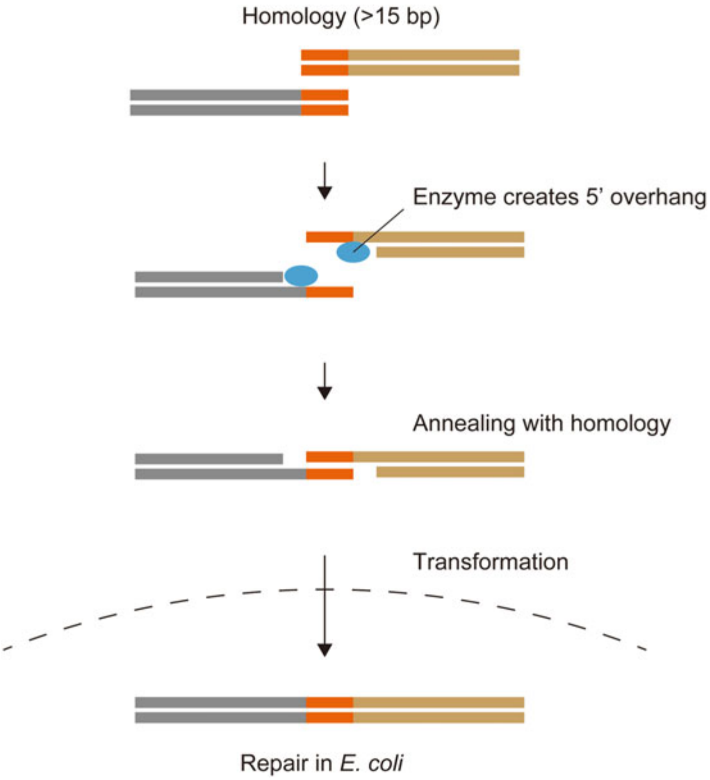

Fig. 3 Principle of the In-Fusion cloning system

high efficiency of cloning long fragments but also the ease of experimental design [16].

2 Materials

2.1 DNA Extraction

1. Mice to be genotyped (*see* **Note 1**).
2. DNA lysis buffer: 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), and 10% sodium dodecyl sulfate (SDS) (*see* **Note 2**).
3. Proteinase K solution, 20 mg/mL.
4. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
5. Heat block equipment.
6. Microvolume spectrophotometer, e.g., NanoDrop One (Thermo Scientific).

2.2 In Vitro Cre Recombination

1. High-efficiency DNA polymerase (*see* **Note 3**).
2. Cre recombinase (NEB).
3. Primer set 1 for the first PCR. These primers should flank the floxed region. The forward primer (FP1) should be located

500–1000 bp upstream of the first loxP site, and the reverse primer (RP1) should be located 500–1000 bp downstream of the second loxP site (*see* Fig. 2).

4. Primer set 2 for the second PCR. These primers should flank the floxed region but be located within the amplicon for the first primer set. The forward primer (FP2) should be located 10–250 bp upstream of the first loxP site, and the reverse primer (RP2) should be located 10–250 bp downstream of the second loxP site. Product size from Cre recombined samples should be less than 500 bp (*see* Fig. 2).
5. Agarose gel.
6. DNA staining solution, e.g., ethidium bromide.
7. Thermal cycler.
8. Electrophoresis chamber.
9. Gel imaging system.

2.3 Cloning of Floxed Regions and Sequencing Analysis

1. High-fidelity DNA polymerase (*see* **Note 4**).
2. In-Fusion HD Cloning Kit (Takara).
3. DNA purification kit.
4. pUC19 cloning vector.
5. Competent cells for transformation of cloning vector.
6. LB agar with ampicillin: 1.5% agar, 1% tryptone, 0.5% yeast extract, 1% NaCl, and 50 μg/mL ampicillin.
7. Primer set 3 to allow amplification of linearized vector. The 5′ ends of each primer should contain 15 bases that are homologous to 15 bases at either end of the insert DNA that is amplified with primer set 1 (*see* **Note 5** and Fig. 4). Forward: 5′- XXXXXXXXXXXXXXXGATCCTCTAGAGTCGACCTG - 3′, and reverse: 5′- YYYYYYYYYYYYYYYGATCCCCGGGTACCGAG-3′. X and Y indicate homology sequences at each end of the insert. Overview of primer design is illustrated in Fig. 4.
8. M13 Forward primer (−20): 5′- GTAAAACGACGGCCAG-3′.
9. M13 Reverse primer: 5′- CAGGAAACAGCTATGAC-3′.

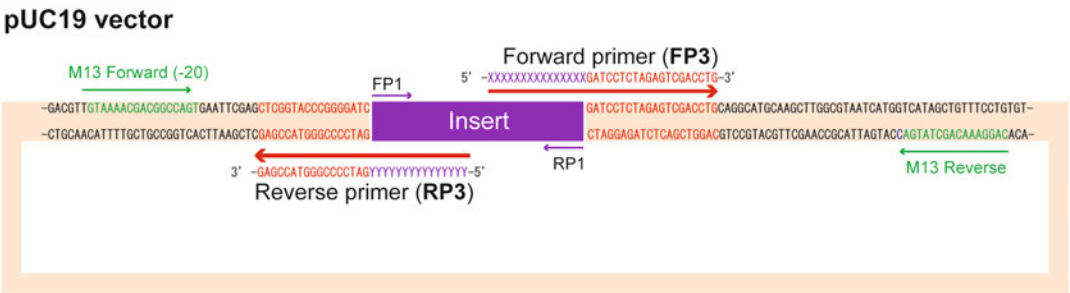
pUC19 vector
M13 Forward (-20)
FP1
Forward primer (FP3)
5' -XXXXXXXXXXXXXXXGATCCTCTAGAGTCGACCTG-3'
-GACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATC
Insert
GATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGT-
-CTGCAACATTTTGCTGCCGGTCACTTAAGCTCGAGCCATGGGCCCCTAG
CTAGGAGATCTCAGCTGGACGTCCGTACGTTCGAACCGCATTAGTACCAGTATCGACAAAGGACACA-
3' -GAGCCATGGGCCCCTAGYYYYYYYYYYYYYYY-5'
Reverse primer (RP3)
RP1
M13 Reverse

Fig. 4 Sequencing map of pUC19 vector and primers for cloning of floxed region. Orange indicates the pUC19 vector and purple indicates the insert of interest (floxed region)

3 Methods

3.1 DNA Extraction from Mouse Tail Samples

1. Cut 5 mm of mouse tail and place into a 1.5 mL tube (*see* **Note 1**).
2. Add 200 μL of DNA lysis buffer and 2 μL of 20 mg/mL Proteinase K solution.
3. Incubate the tube overnight at 55 °C.
4. Put the tube at 85 °C for 45 min to inactivate proteinase K.
5. Purify DNA by phenol/chloroform extraction and ethanol precipitation.
6. Calculate the concentration of purified DNA and store at −20 °C until use.

3.2 In Vitro Cre Recombination

All procedures are performed on ice unless otherwise stated.

3.2.1 First PCR for Amplification of Floxed Region

1. Perform PCR amplification of full length of the floxed region using 10 ng of tail DNA from Subheading 3.1, above, and primer set 1, in a 20 μL reaction volume (*see* **Note 6**).
2. To confirm amplification, analyze 3 μL of PCR product by agarose gel electrophoresis.

3.2.2 In Vitro Cre Recombination of PCR Fragments

1. Perform in vitro Cre recombination of PCR products. Add the following to 0.2 mL PCR tubes on ice: 1 μL of first PCR product, 1 μL of 10× Cre recombinase buffer, and two units of Cre recombinase. Adjust volume to 10 μL with nuclease-free water (*see* **Note 7**).
2. Incubate at 37 °C for 30 min, and then inactivate Cre recombinase by incubation at 70 °C for 10 min.

   Keep samples on ice or store at −20 °C.

3.2.3 Second PCR for Detection of Recombined Fragments

1. Perform the second PCR in 10 μL reaction volume using primer set 2 and 1 μL of Cre recombinase reaction mix as template for Cre-plus sample. Dilute PCR products from the first PCR 1:10, and use as template for Cre-minus control sample (*see* **Note 8**).
2. Analyze 3 μL of PCR products by agarose gel electrophoresis (*see* **Notes 9–11**). The presence of a small PCR product of the appropriate size, as determined by primer set 2, specifically in the Cre-plus sample but absent from the Cre-minus sample, will indicate mice with functional floxed alleles. Samples with unintended alleles do not show such amplification (*see* **Note 12** and Fig. 5 for example data).

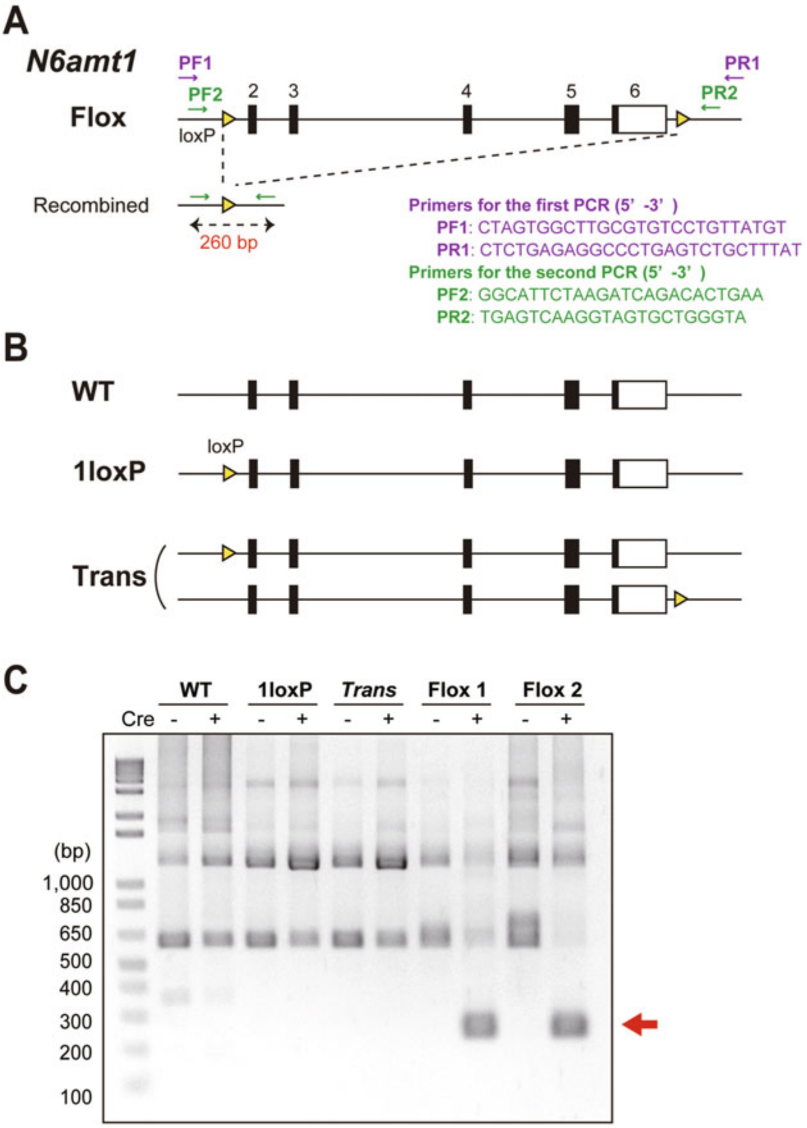
A
N6amt1
PF1
PR1
PF2
PR2
2
3
4
5
6
Flox
loxP
Recombined
260 bp
Primers for the first PCR (5' -3')
PF1: CTAGTGGCTTGCGTGTCCTGTTATGT
PR1: CTCTGAGAGGCCCTGAGTCTGCTTTAT
Primers for the second PCR (5' -3')
PF2: GGCATTCTAAGATCAGACACTGAA
PR2: TGAGTCAAGGTAGTGCTGGGTA
B
WT
loxP
1loxP
Trans
C
WT
1loxP
Trans
Flox 1
Flox 2
Cre
- + - + - + - + - +
(bp)
1,000
850
650
500
400
300
200
100

Fig. 5 Model experiments for *N6amt1*-flox mouse detection. Map of floxed region of *N6amt1*-flox allele (**a**) and potential unintended alleles (**b**). Gel image of the second PCR products for mice possessing *N6amt1*-flox or unintended alleles (**c**). Red arrow indicates a specific amplification from the floxed allele-derived sample

3.3 Cloning of Floxed Regions and Sequencing Analysis

3.3.1 Preparation of Linearized Vector by Inverse PCR

1. Amplify linearized vector using high-fidelity DNA polymerase with primer set 3, which contain 15 bp regions homologous to the ends of the insert. Use 1 ng of pUC19 vector as the PCR template.
2. Analyze PCR product by agarose gel electrophoresis.
3. Purify PCR product using an appropriate DNA purification kit.
4. Calculate the concentration of purified DNA and store at −20 ° C until use.

3.3.2 Preparation of Insert

1. Perform PCR amplification of the full length of the floxed region with primer set 1 from 10 ng of tail DNA of candidate flox mice identified in Subheading 3.2.
2. To confirm amplification, analyze 3 μL of PCR product by gel electrophoresis.
3. Purify PCR product using an appropriate DNA purification kit.
4. Calculate the concentration of purified DNA and store at −20 ° C until use.

3.3.3 Cloning and Sequencing

1. Mix 2 μL of 5× In-Fusion HD Enzyme Premix, linearized vector, and insert and adjust volume to 10 μL using nuclease-free water. Molar ratio of vector to insert should be 1:2 (*see* **Note 13**). Total DNA amount should be less than 400 ng.
2. Incubate at 50 °C for 15 min.
3. Perform bacterial transformation by adding 1 μL of the reaction mix into 20 μL of competent *E. coli*. Plate the transformation mix onto LB agar plates with ampicillin. Incubate plates overnight at 37 °C.
4. The following day, pick at least ten colonies, and screen for the presence of the floxed fragment by in vitro Cre recombination as described in Subheading 3.2, but using M13 Forward (−20) and M13 Reverse primers for the first PCR reaction.
5. Confirm sequences of colonies with the floxed fragment by Sanger sequencing.

4 Notes

1. We cut tails of mice at the time of weaning (3 weeks after birth).
2. We often use DirectPCR Lysis Reagent (Viagen Biotech) in place of lysis buffer prepared in house. If this reagent is used, the DNA purification step (phenol/chloroform extraction and ethanol precipitation) may be omitted. For the first PCR, dilute lysis reagent 1:20 and use 1 μL as PCR template.
3. Tks Gflex DNA polymerase (Takara) works well for the first and second PCR in our laboratory. For amplification of PCR products longer that 5000 bp, we prefer KOD FX Neo DNA Polymerase (Toyobo) (also *see* **Note 6**).
4. PrimeSTAR GXL DNA Polymerase (Takara) works well for amplification with high-fidelity.
5. To improve DNA amplification efficiency, homology arms should be put in PCR primers used to amplify the vector DNA, but should be omitted from primers for the insert DNA. The presence of these additional arms on primers may interfere with precise amplification from genomic DNA.

6. It is important to obtain the correct PCR product here. To avoid nonspecific amplification, primers and PCR conditions should be optimized. For amplification of PCR products greater that 5000 bp, we perform two-step cycle PCR using KOD FX Neo DNA Polymerase (Toyobo) and 25–30 mer primers with Tm of 65–67 °C. Online tools such as Primer3 (https://primer3.org) are useful for primer design. Example condition of two-step cycle PCR: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 98 °C for 10 sec, and elongation at 68 °C for 1 min/kb.
7. We found that Cre recombinase easily lost its enzymatic activity if not handled carefully. Return the enzyme to freezer immediately after use and do not use it beyond the expiration data.
8. To ensure specific amplification from recombined fragments, the extension step in the second PCR program should be set to 30 s or shorter. Example PCR conditions when using Tks Gflex DNA Polymerase are initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 68 °C for 30 s.
9. Additional nonspecific bands are often observed (Fig. 5c).
10. If there is a deletion allele in the genome, a small band will also appear in Cre-minus control samples.
11. Mutations in loxP sites will affect recombination efficiency. However, we observed successful recombination even with a 5 bp mismatch mutation in loxP.
12. An example showing screening of flox mice for *N6amt1* gene (RefSeq accession no. NM_026366) is shown in Fig. 5. *N6amt1*-flox mice were generated by insertion of two loxP sites flanking exons 2–6 of *N6amt1* gene by CRISPR-Cas9 genome editing (Fig. 5a). As sample sets contain not only flox mice but also mice with unintended-edited alleles, such as knock-in of only one loxP (1loxP) or of two loxP in *trans* (Fig. 5b), we demonstrate that this method can efficiently identify floxed animals (Fig. 5c).
13. If insert size is greater than 10,000 bp, the molar ratio of vector to insert should be between 1:1 and 2:1.

Acknowledgment

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Chapter 13

VCre/VloxP and SCre/SloxP as Reliable Site-Specific Recombination Systems for Genome Engineering

Manabu Nakayama

Abstract

The Cre/loxP system is a versatile and powerful tool that has been used to develop many kinds of genetically modified mice, such as conditional knockout mice and mutant protein-expressing mice through the excision of a STOP cassette. However, while numerous in vivo and in vitro applications of the Cre/loxP system have been reported, it remains difficult to target at one time more than one set of recognition sites in an identical single cell in mice using the Cre/loxP system. To overcome this barrier, we developed two novel site-specific recombination systems called VCre/VloxP and SCre/SloxP. These systems allow multiple independent site-specific recombination, for example, multiple targeted deletions in the same cell at different times. In this chapter, I describe the features of VCre/VloxP and SCre/SloxP, practical protocols and tips on how to use them in genomic engineering applications, potential problems in their use, and how problems can be identified and solved.

Key words Site-specific recombination, Genome engineering, Cre/loxP, Flp/FRT, Conditional knock-out mice, Knock-in, SSR (site-specific recombinase)

1 Introduction

The Cre/loxP system is the most popular system for site-specific recombination. This system comprises a Cre recombinase that recognizes a 34 bp DNA sequence called loxP site. Cre recombinase mediates the deletion of DNA sequences between two loxP sites when the orientation of the two loxP sites are the same; however, if the two lox P sites have the opposite orientation, Cre recombinase mediates the inversion of DNA sequences between them [1].

Many methods in molecular genetics based on the Cre/loxP system continue to be developed. For example, researchers have used Cre/loxP site-specific mutagenesis to create conditional knockout mice by manipulating gene expression through the excision of exon(s) flanked by loxP sites in mouse genome [2]. By crossbreeding tissue-specific-expressing Cre mice or inducible Cre

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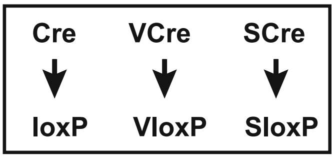

Fig. 1 Schematic showing Cre, VCre, and SCre recombinases and their recognition sites. Cre, VCre, and SCre specifically recombine the loxP, VloxP, and SloxP sites, respectively. Cre/loxP, VCre/VloxP, and SCre/SloxP do not cross-react with each other

mice, researchers can determine the impact of knocking out a gene of interest in specific tissue or the impact of knocking out a gene in a time-specific manner. In conditional knock-in mice, a mutant protein of interest can be expressed through the excision of a STOP cassette. Other valuable Cre/loxP-based methods and applications have been developed, such as recombinase-mediated cassette exchange (RMCE) [3], Brainbow cell lineage analysis [4], FLEx switch irreversible inversion [5], and viral vector packaging [6]. However, conflicts arise when using the multiple recombining methods simultaneously in the same system. Due to cross-reactivity, one cannot use more than one Cre/loxP within the same construct.

To address this cross-reactivity problem, about a decade ago, we developed two novel site-specific recombination systems called VCre/VloxP and SCre/SloxP that recognize VloxP and SloxP, sites that differ from loxP and FRT sites (recognition sites for another widely used site-specific recombinases Cre and Flp, respectively) (Fig. 1) [7]. We also developed mutant VloxP (Vlox2272) and SloxP (Slox2272), which can be used the same way as mutant loxP (Fig. 2a, b). These two novel recombination tools can be used in cultured cells and mouse embryonic stem (ES) cells and also in genetically modified mice [8], fish (*Oryzias latipes*) [9], *Escherichia coli* [10], and retroviruses [11].

VCre recombinase and its recognition site, VloxP, originate from plasmid p0908 of *Vibrio* sp.; VCre recombinase recombines VloxP sites. SCre recombinase and its recognition site, SloxP, originate from plasmid 1 of *Shewanella* sp. ANA-3; SCre recombinase recombines SloxP sites. VloxP and SloxP recognition sites differ from loxP. Moreover, VCre/VloxP, SCre/SloxP, and Cre/loxP do not cross-react with each other (Fig. 1). As shown in Fig. 2, loxP, VloxP, and SloxP are 34 bp (13-8-13) sequences that consist of two 13 bp inverted repeats and 8 bp core sequences. The DNA sequence structures of their recognition sites are identical. The 8 bp core sequence is important for determining the orientation of recognition sites like the loxP site. While loxP is a perfect 13 bp inverted repeat, VloxP harbors one mismatch in its 13 bp inverted repeat. When we created a perfect 13 bp inverted repeat for VloxP

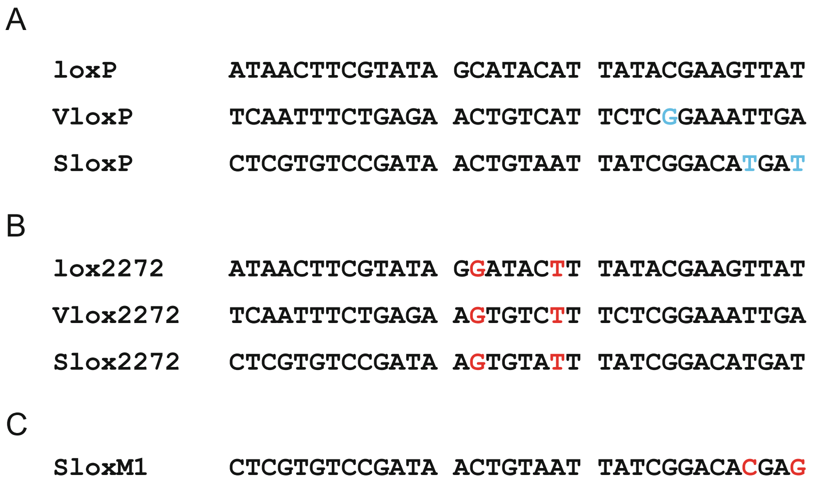
A
loxP ATAACTTCGTATA GCATACAT TATACGAAGTTAT
VloxP TCAATTTCTGAGA ACTGTCAT TCTCGGAAATTGA
SloxP CTCGTGTCCGATA ACTGTAAT TATCGGACATGAT
B
lox2272 ATAACTTCGTATA GGATACTT TATACGAAGTTAT
Vlox2272 TCAATTTCTGAGA AGTGTCTT TCTCGGAAATTGA
Slox2272 CTCGTGTCCGATA AGTGTATT TATCGGACATGAT
C
SloxM1 CTCGTGTCCGATA ACTGTAAT TATCGGACACGAG

Fig. 2 Sequence comparisons of respective recognition sites of Cre, VCre, and SCre recombinases. (**a**) Comparison of loxP, VloxP, and SloxP sites. Light blue-colored bases indicate mismatch in the 13 bp inverted repeats. (**b**) Comparison of three mutant sites of loxP, VloxP, and SloxP (lox2272, Vlox2272, and Slox2272, respectively). These mutations contain two base substitutions (C-to-G substitution at position no. 2; A-to-T substitution at position no. 7) in the 8 bp core sequence of loxP, VloxP, and SloxP. These substitutions are indicated by red-colored bases. (**c**) DNA sequence of SloxM1. Because the SloxP site contains two base-pair mismatches in its 13 bp inverted repeats, an unexpected fusion protein can be produced through the inadvertent matching of coding sequence frame. For this reason, we used the SloxM1 site (and also call it SloxP as general term) for the SCre/SloxP system, as the 13 bp inverted repeats of the SloxM1 site were designed to perfectly match. These substitutions are indicated by red-colored bases

by constructing two mutants, this mismatch did not change its recombination efficiency [7]. Thus, the presence of this mismatch should not be of concern in terms of VCre/VloxP's recombination efficiency.

SloxP is composed of an 8 bp sequence flanked on either side by 13 bp inverted repeats. Unlike loxP, the two 13 bp segments of SloxP do not perfectly match because of a two-base mismatch (Fig. 2a). We modified SloxM1 (CTCGTGTCCGATA ACTGTAAT TATCGGACACGAG) so that the two 13 bp segments would match perfectly. Fortunately, the modified SloxM1 does not contain an ATG, which can be used as an initial Met codon (Fig. 2c). LoxP flanked STOP cassette is often located just in front of the initial Met codon (ATG) of a gene of interest and is used to modulate expression through the excision of the STOP cassette via Cre. When an ATG-containing SloxP is positioned in front of a Met codon in the same way loxP as usually positioned, an unexpected fusion protein can be produced through the accidental matching of coding sequence frame. For these reasons, we chose SloxM1 for use in our SCre/SloxP recombination system. SloxM1 (we also call it the SloxP site as a general term) contains a perfectly matched 13 bp inverted repeat but does not contain an ATG in any of the three frames. It is often used in our experiments.

Mutant loxP sites can be leveraged for genomic engineering, significantly increasing the overall utility of the Cre/loxP system. Previous analyses of loxP mutants demonstrated that the 8 bp core sequence directs the orientation and convertibility of mutant loxP sites [12]. Two base substitutions in the 8 bp sequence of VloxP were made to form mutant Vlox2272. Vlox2272 possesses a C-to-G substitution at position no. 2 and an A-to-T substitution at position no. 7. We named this mutant Vlox2272, because its base substitutions were analogous to those of lox2272 and because two other substitutions in the 8 bp sequence of Vlox2272 matched that of lox2272 (Fig. 2b).

VloxP and Vlox2272 do not cross-react, even though VCre recombines both sites. In addition to mutant VloxP (Vlox2272), we also designed a mutant SloxP, called Slox2272, which contains two base substitutions (C-to-G substitution at position no. 2; A-to-T substitution at position no. 7) in its 8 bp core sequence. We named this mutant Slox2272, because its base substitutions were analogous to those of lox2272 and because two other substitutions in the 8 bp core sequence of Slox2272 matched that of lox2272 and Vlox2272 (Fig. 2b). SloxP and mutant Slox2272 do not cross-react, even though SCre recombines both sites. As with other previously developed mutant sites, Vlox2272 and Slox2272 can be used as useful mutant sites like the lox2272 site in Cre/loxP system.

The VCre/VloxP and SCre/SloxP system can be used not only in cultured cells and *E. coli* but also in genetically modified mice. Previously, we established four novel knock-in (KI) mouse strains in which VloxP-STOP-VloxP-EGFP, SloxP-STOP-SloxP-tdTomato, CAG-VCre, and CAG-SCre genes were inserted into the ROSA26 locus [8]. VloxP-STOP-VloxP-EGFP and SloxP-STOP-SloxP-tdTomato KI mice are reporter mice carrying EGFP or tdTomato genes posterior to the STOP cassette, which is floxed by VloxP or SloxP sites, respectively. CAG-VCre and CAG-SCre KI mice carry VCre or SCre genes that are expressed ubiquitously via a CAG promoter. We crossed these two reporter mice with three different deleter mice (CAG-VCre KI, CAG-SCre KI, or Cre-expressing transgenic mice). These matings demonstrated that VCre/VloxP and SCre/SloxP functioned similarly to Cre/loxP in mice and that the two recombinases exhibited tight specificity for their recognition sequences. We also demonstrated that each recombinase was highly specific for its respective target sites and that VCre/VloxP, SCre/SloxP, and Cre/loxP did not cross-react with each other. Figure 3 illustrates in detail how VCre-expressing mice and reporter mice for VCre/VloxP detection were produced. In VCre-expressing mice, ubiquitous VCre expression was directed by a strong CAG promoter. In VCre reporter mice, a STOP cassette flanked by two VloxP sites was positioned between the CAG promoter and EGFP gene. The STOP cassette, which contains a stop

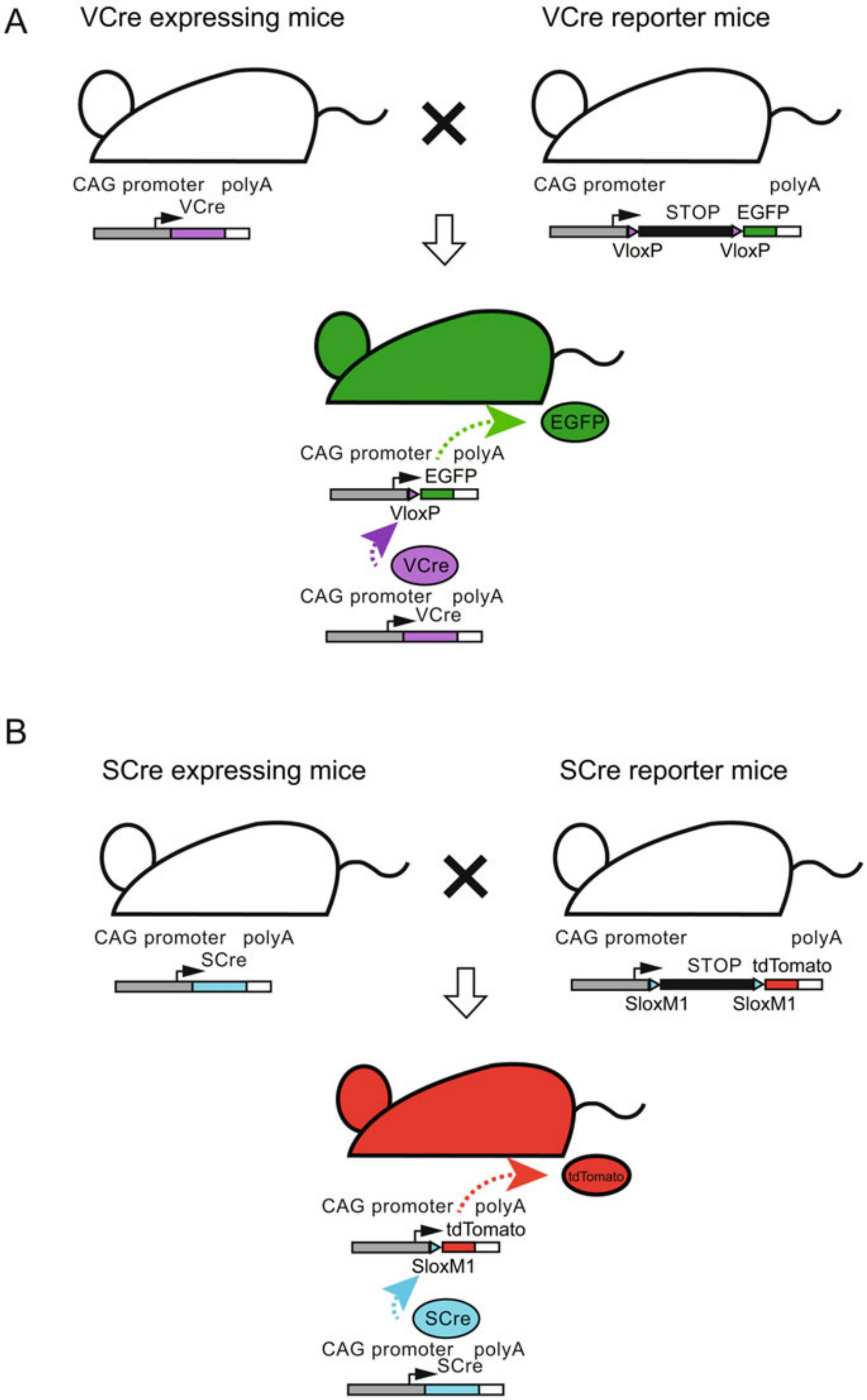

Fig. 3 Schematic diagram showing the creation of VCre-expressing mice and their reporter mice. (**a**) VCre-expressing mice were created by knock-in of the CAG promoter, VCre gene, and polyA additional signal (rabbit beta-globin polyA) into the ROSA26 locus. The CAG promoter directs the ubiquitous expression of the VCre gene in these mice. Next, VCre reporter mice were created by knock-in of the CAG promoter, STOP cassette flanked by two VloxP sites, EGFP gene, and polyA additional signal (rabbit beta-globin polyA) into the ROSA26 locus. The STOP cassette prevented the expression of the EGFP gene transcriptionally and translationally.

codon and a polyA additional signal, can block the expression of the EGFP gene via the CAG promoter. When VCre-expressing mice and their reporter mice were crossbred, the resulting pups ubiquitously expressed EGFP protein after excision of the STOP cassette via the VCre/VloxP reaction. A similar scenario occurred with SCre-expressing mice and SCre reporter mice (Fig. 3b). When SCre-expressing mice and their reporter mice were crossbred, the resulting pups ubiquitously expressed fluorescent tdTomato protein after the excision of the STOP cassette via the SCre/SloxP reaction.

Site-specific recombination consists of multiple steps, namely, recognition of a specific DNA site; formation of covalent 3′--phophotyrosine linkages to the DNA substrate; ligation of DNA; and, after Holliday junction formation, repeated cutting and ligation of DNA. While VCre and SCre are ~30% identical to the amino acid sequences of Cre recombinase, the structure and the amino acid sequences of their active center for site-specific recombination are almost identical. Although these proteins recognize distinct recognition sites, their function is basically identical. Many recombination studies have concluded that the site-specific recombination reaction of Cre and Flp recombinases begins with the cleavage of the recombining sites at tyrosine residues to form covalent 3′--phophotyrosine linkages to the DNA substrates in the recognition site and the subsequent release of free 5′-hydroxyl groups, indicating that this tyrosine residue is essential for catalysis [13, 14]. Substitution to leucine at Tyr349 of VCre and Tyr352 of SCre demonstrated that these tyrosine residues are also essential for site-specific recombination. Interestingly, the H289L mutant form of Cre, which corresponds to H314L of VCre, resulted in the accumulation of covalent intermediates. In fact, the H314L mutant of VCre exhibits not only a significant decrease in the efficiency of site-specific recombination but also a low recovery of colony formation. This phenomenon suggests that a plasmid linked covalently by VCreH314L cannot replicate normally in *E. coli*

Fig. 3 (continued) VCre-expressing mice were then mated with VCre reporter mice. The resulting pups harbored both alleles and were able to express the EGFP gene after the excision of the STOP cassette by the VCre/VloxP reaction. The bodies of these pups fluoresce (green) due to the ubiquitous expression of EGFP when incident light from a trans-illuminated epifluorescence microscope or a fluorescence light box illuminates the body. (**b**) SCre-expressing mice were created by knock-in of the CAG promoter, SCre gene, and polyA additional signal (rabbit β-globin polyA) into the ROSA26 locus. The CAG promoter directs the ubiquitous expression of SCre gene. Next, SCre reporter mice were created by knock-in of the CAG promoter, STOP cassette flanked by two SloxM1 sites, tdTomato gene, and polyA additional signal (rabbit β-globin polyA) into the ROSA26 locus. The STOP cassette prevented the expression of the tdTomato gene transcriptionally and translationally. SCre-expressing mice were then mated with SCre reporter mice. The resulting pups harbored both alleles and were able to express the tdTomato gene after the excision of the STOP cassette by the SCre/SloxP reaction. The bodies of these pups fluoresce (red) due to the ubiquitous expression of tdTomato

DH10B lacking the RecA enzyme. This phenomenon is also observed in mutant H317L of SCre. Taken together, these analyses of mutants suggest that VCre, SCre, and Cre recombine DNA via the same mechanism but using different recognition sites, even though the amino acid sequences of these three recombinases are less identical to each other. These results support the possibility that VCre/VloxP and SCre/SloxP can be reliably used in various methods that employ the Cre/loxP system, because all three recombinases (VCre, SCre, and Cre) have almost the same structure and the same mechanism for site-specific recombination [10].

To date, we have successfully used VCre/VloxP and SCre/SloxP for site-specific recombination in mice [8, 15], fish (*O. latipes*) [9], *E. coli* [10], and retroviruses [11]. Importantly, we have also successfully used VCre/VloxP and SCre/SloxP for introducing artificial human chromosomes into ES cells [7, 16] and for altering bacterial artificial chromosomes (BACs) [10]. In addition to collaborative work done with our laboratory, other independent groups have also used the VCre/VloxP and SCre/SloxP site-specific recombination systems. For example, Kim et al. [17] conducted mosaic analyses using dual recombinase-mediated cassette exchange (MADR), which permits stable labeling of mutant cells expressing transgenic elements from precisely defined chromosomal loci. Their experiments demonstrated that three useful site-specific recombinases are now available for use: VCre recombinase in addition to Cre and Flp, two other commonly used recombinases.

Another technique that uses VCre recombinase is intronic recombinase sites enabling combinatorial targeting (INTRSECT) [18]. Fenno et al. [18] developed INTRSECT to express adeno-associated virus (AAV)-borne molecular “payloads” in cells. INTRSECT is based on two genetically and/or anatomically defined parameters by placing two orthogonal recombinase (Cre and Flp) recognition sequences in synthetic introns. Fenno et al. [19] also developed the first versatile single-virus tool (coined Triplesect) that enables targeting of cell types that are defined by three parameters. The recombinases used in the Triplesect method were VCre, Cre, and Flp. Fenno et al. concluded: “VCre, in contrast, was orthogonal to all of the tested recombinases (no indication of cross-activity) and, thus, found to be potentially best-suited for use in parallel with Cre and Flp” [19].

A similar method termed the Boolean logic and arithmetic through DNA excision (BLADE) platform was developed by Weinberg et al. [20] as a single-layer platform for BLADE in mammalian cells. They used VCre, VloxP, and Vlox2272 sites in three-input BLADE. Weinberg et al. [21] also compiled a library of high-performance chemical- and light-inducible recombinases for use in cultured mammalian cells and in vivo mice, employing an inducible split VCre system. They created chemical-inducible dimerization (CID) domains of VCre, comprising a N-terminal split half and C-terminal split half. CID systems are advantageous, because split proteins tagged with CIDs can be rendered nonfunctional until a small molecule inducer is administered. This split VCre system was tested in three inducible systems (rapamycin, phytohormone abscisic acid [ABA], and phytohormone gibberellin [GIB]). Addition of a small molecular inducer produced the expected response, but optimization is still needed.

Bessen et al. [22] reported a new method for revealing the DNA specificity of site-specific DNA recombinases with single-nucleotide resolution; they call it Rec-seq. With Rec-seq, high-throughput DNA sequencing (HTS) is used in a comprehensive and unbiased manner. For validation, they applied Rec-seq to characterize the DNA specificity of some site-specific DNA recombinases containing VCre. Rec-seq profiling of VCre suggests several important base positions in the 34 bp of the VloxP site.

The site-specific recombinase VCre can also be used to study biosynthetic pathways in vitro. After purifying VCre by nickel-affinity chromatography, Liu et al. [23] showed that VCre is capable of reacting with its respective conditional recombination site in vitro. On the basis of that finding, Liu and colleagues developed a one-step method for optimizing biosynthetic pathways of interest by incubating a mixture of DNA with the VCre recombinase system.

This brief overview of the VCre/VloxP and SCre/SloxP systems and their various applications in diverse areas of genomic engineering shows that these site-specific recombinases have great potential for use in more complicated and fine genome engineering manipulations. For the remainder of this chapter, I present practical protocols and tips on how to use the VCre/VloxP and SCre/SloxP systems to create VCre-expressing mice, harboring a knock-in gene of interest in a genome that is expressed in a tissue-specific pattern. I also discuss potential problems with this method and how these problems can be identified and addressed.

2 Materials

2.1 Pertinent Sequence Information

1. VCre DNA sequence data (GenBank/EMBL/DDBJ, accession number: AB618652).
2. SCre DNA sequence data (GenBank/EMBL/DDBJ, accession number: AB618653).

2.2 Locations of VCre Plasmids Containing VCre or SCre

1. pUCNLS-VCre (SSR00001, Center for Development of Biotech Industries at Kazusa DNA: https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr.html).

2. The DNA sequence of the pUCNLS-VCre can be found at https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr_data/pUCNLS-VCre.html.
3. The map of the pUCNLS-VCre plasmid can be found at https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr_data/pUCNLS-VCre-map.pdf.
4. pUCNLS-SCre (SSR00002, the Center for Development of Biotech Industries at Kazusa DNA Research Institute: https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr.html).
5. The DNA sequence of pUCNLS-SCre can be found at https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr_data/pUCNLS-SCre.html.
6. The map of pUCNLS-SCre can be found at https://www.biosupport.kazusa.or.jp/english/sub_center1/ssr_data/pUCNLS-SCre-map.pdf.

2.3 DNA Sequence of Targeting Vectors

1. The DNA sequences of the targeting vectors for VCre-expressing mice, VCre-reporter mice, SCre-expressing mice, and SCre-reporter mice can be found in the electronic supplementary material of Yoshimura et al. (https://link.springer.com/article/10.1007%2Fs11248-018-0067-0#Sec16) [8].

2.4 Useful Plasmids

1. pTurboVCre [7]: This plasmid is used to express VCre protein in cultured cells.

   pTurboVCre constitutively produces VCre recombinase from a CAG promoter composed of a CMV enhancer upstream from a chicken β-actin promoter, as described previously [7].
2. NLS-FLAG-VCre: This plasmid is composed of a SV40 NLS and a FLAG sequence introduced into the N terminus of the VCre CDS. The artificially synthesized VCre genes are optimized for mouse codon usage in addition to removing all CpG dinucleotides.

   pTurboSCre [7]: This plasmid is used to express SCre protein in cultured cells.
3. pTurboSCre is constructed by replacing the NLS-FLAG-VCre gene with NLS-FLAG-SCre.

   pSIMVCre [7]: This plasmid is used to express VCre protein in *E. coli*.
4. Plasmid pSIMVCre contains a temperature-sensitive, low-copy plasmid derived from pSC101 and carries an expression system under the control of a lambda pL promoter, a cI857 temperature-sensitive repressor, and a hygromycin-resistance gene. As this plasmid is usually maintained at 32 °C, heat-induced expression of VCre protein should occur at 42 °C. Specifically, *E. coli* DH10B harboring the expression plasmid pSIMVCre, which encodes VCre, is grown at 32 °C overnight

in Luria-Bertani (LB) medium containing 75 μg/mL of hygromycin on a shaker at 170 rpm. Fifty microliters of the overnight culture are inoculated into a 2 mL fresh LB medium containing 75 μg/mL of hygromycin. The culture is incubated at 32 °C for 2 h on a shaker, heated in a 42 °C water bath for 15 min, and then chilled on ice for 5 min. For electroporation, cells are washed three times with cold water and 10% glycerol, and electroporated with a tester plasmid (0.2 μg) in 25 μL 10% glycerol. The tester plasmid contains an additional antibiotics resistance gene flanked by two VloxP sites. One milliliter of super optimal broth with catabolite repression (SOC) medium is added to the transformation mixture. Transformants are incubated at 37 °C for 2 h and then plated onto an LB agar plate containing appropriate antibiotics.

5. pSIMSCre [7]: This plasmid is used to express SCre protein in *E. coli*.

   pSIMSCre is constructed by replacing the NLS-FLAG-VCre gene with NLS-FLAG-SCre.

2.5 Genome Databases

1. Information on various genes of interest can be found at the Ensembl Genome Browser (http://asia.ensembl.org/index.html), UCSC Genome Browser (http://genome.ucsc.edu/), and NCBI Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/).

2.6 DNA Sequence Analysis Software

1. The following software can be used to analyze DNA sequences: Sequencher®

   (Gene Codes Corporation; https://www.genecodes.com/); SnapGene® (https://www.snapgene.com/); A Plasmid Editor (ApE; https://jorgensen.biology.utah.edu/wayned/ape/).

2.7 Online Vector Design and Construction Services

1. VectorBuilder (https://en.vectorbuilder.com/) offers customized vector design and construction services.

2.8 Gene Synthesis Services

1. The following companies offer customized gene synthesis services: Genscript®

   (https://www.genscript.com/); Eurofins (https://www.eurofins.com/genomic-services/).

2.9 BAC Genomic DNA Bioresources

1. Information on BAC clones originating from the genomic DNA of C57BL/6N mice can be found at RIKEN BRC (https://dna.brc.riken.jp/en/).
2. Information on BAC clones originating from the genomic DNA of C57BL/6J mice can be found at BACPAC Resources Center (BPRC; https://bacpacresources.org/) or at ThermoFisher Scientific (https://clones.thermofisher.com/cloneinfo.php?clone=bacpac).

3 Methods

In this section, we describe how to use VCre to make tissue-specific or cell-specific VCre-expressing mice. This procedure involves making a targeting vector that will be used to knock-in a gene of interest into the mice. Because VCre, SCre, and Cre recombine DNA through the same mechanism, using different recognition sites, VCre-expressing and SCre-expressing mice can be prepared using the same protocol as that used for preparing Cre-expressing mice.

3.1 Designing the Targeting Vector

In this procedure, traditional homologous recombination using mouse ES cells will be performed but not by using knock-in with CRISPR/Cas9 (*see* **Note 1**) (Fig. 4).

1. Examine the exon/intron organization of the gene of interest in the Ensembl Genome Browser (*see* **Note 2**).
2. For the gene of interest, select the major form of its transcript. If tissue-specific transcripts are expressed via a special promoter, select the tissue-specific promoter of interest (*see* **Note 3**).
3. Place next to the initial Met ATG codon of the gene of interest with the ATG of VCre gene by using DNA analysis software (*see* **Notes 4**, **5**, **6**, and **7**).
4. Insert the DNA sequence of the polyA additional signal downstream of the VCre gene at the DNA sequence level (*see* **Note 8**).
5. Insert the FRT-Neo-FRT cassette (positive selection marker) downstream of the polyA additional signals at the DNA sequence level (*see* **Note 9**).
6. Arrange the DNA sequence of the homology arms on both sides (*see* **Notes 10** and **11**).
7. Insert the DNA sequence of DT-A expression cassette (negative selection marker) (*see* **Note 12**).
8. Insert the site of restriction endonuclease, NotI. When the plasmid containing the targeting vector is introduced into ES cells by electroporation, the plasmid should be single-cut and linearized. Thus, the NotI site should be positioned to enable a single-cut in the whole plasmid. If the DNA sequence of the homology arms contains the NotI site, change the location of the NotI site so it is a unique cutter (*see* **Note 13**).
9. Finally, design the targeting vector for knock-in of the VCre gene as shown in Fig. 4. Confirm the DNA sequence of the targeting vector. The plasmid sequence should also contain a replication origin of the plasmid and an antibiotic resistance gene for *E. coli*.

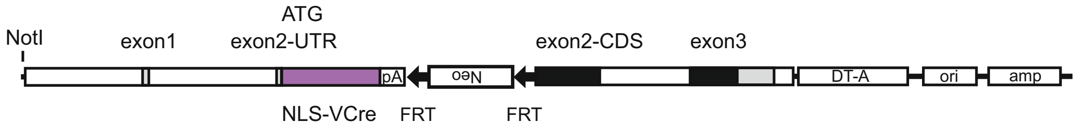

Fig. 4 Schematic diagram of a targeting vector of VCre-expressing mice for knocking in a gene of interest. VCre gene with NLS (nuclear localization signal), polyA additional signal (SV40 polyA), reverse-oriented Neo expression cassette flanked by two FRT sites (positive selection marker) were inserted to match the initial Met codon (ATG) of the gene of interest. The targeting vector contains two homologous arms (left arm and right arm), a DT-A expression cassette (negative selection marker), replication origin for plasmid, and an ampicillin resistance gene. The targeting vector also contains a unique restriction endonuclease (NotI site). NLS-VCre VCre gene with nuclear localization signal (NLS), pA SV40 polyA additional signal, Neo Neo expression cassette (mouse PGK promoter, Neomycin-resistance gene and artificial polyA additional signal), DT-A DT-A expression cassette (mouse PGK promoter, diphtheria toxin A subunit gene, and artificial polyA additional signal), ori replication origin for plasmid, amp ampicillin-resistance gene with bacterial promoter, exon2-UTR untranslated region of exon2, exon2-CDS coding sequence of exon2

10. Construct the targeting vector using the standard methods. Conventional ligation methods, PCR ligation, or the double Red recombination method can be used for constructing the targeting vector (*see* **Notes 14**, **15**, **16**, **17**, **18**, **19**, and **20**). Alternatively, one can choose to outsource the construction of the vector to VectorBuilder or other artificial gene synthesis companies.

4 Notes

1. The VCre gene can be inserted into the gene of interest by using CRISPR/Cas9, because VCre and SCre genes are approximately 1 kbp (i.e., 1188 bp and 1161 bp, respectively, including nuclear localization signal (NLS) sequences). Even though knock-in of a foreign gene using CRISPR/Cas9 methods has been reported to have a high success rate [24, 25], researchers can expect that they will still have to perform a trial-and-error step when performing the knock-in procedure with a gene of interest. Although some may think that traditional homologous recombination with mouse ES cells is time-consuming and labor-intensive, in the long term, the total time with this traditional strategy is often shorter than that with CRISPR/Cas9 methods, which have unexpected problems (e.g., off-target effects and not obtaining positive clone). Each step in the traditional homologous recombination procedure is reliable and stable, and one can calculate the time needed for each step. Thus, I strongly recommend the traditional homologous recombination methods in mouse ES cells, especially if one uses a complicated targeting vector.

2. Alternatively, the UCSC Genome Browser or the NCBI Genome Data Viewer can be used.
3. Many gene transcripts have some alternative splicing forms. It is difficult to judge the most suitable initial Met ATG site of a gene, especially, if the gene has many transcriptional start sites and a couple of initial Met ATG sites for translation. In some cases, only one promoter among several promoters in a gene directs tissue-specific expression of interest, while the other promoters direct expression in other tissue. Thus, one should carefully read the literature on the gene of interest to determine which ATG is best in a couple of alternative splicing forms and translational initial Met codons. Unfortunately, the information in popular databases (e.g., Ensembl Genome Browser, UCSC Genome Browser, and NCBI Genome Data Viewer) is not always organized well and rigorously scrutinized. Because these databases still contain information about very rare transcripts and artificial cDNAs produced accidentally during experiments, researchers should pay close attention when identifying which form is the major and biologically significant form. To avoid potential problems, it would be a good idea to pay particular attention to common transcripts while comparing mouse expression data with human expression data. In general, if a gene is studied by many researchers, there will be much data on the expression of that gene, and often the data will be scrutinized and corrected when needed.
4. VCre originates from plasmid p0908 of *Vibrio* sp. SCre originates from plasmid 1 of *Shewanella* sp. ANA-3. The artificially synthesized genes for VCre and SCre were optimized for mouse codon usage. SV40 nuclear localization signal (NLS), MVPKKKRK, was introduced into the N terminus of VCre and SCre CDS because a NLS sequence is needed in order for the genes to move into the nucleus and to make contact with the genomic DNA for effective site-specific recombination to occur. A FLAG sequence, DYKDDDDK, was also introduced into the N terminus of the VCre and SCre CDS as a marker, which can be detected by immunoblotting with commercially available antibodies. The exact order in the N terminus of VCre or SCre is MVPKKKRK-DYKDDDDK.
5. When producing tissue-specific VCre-expressing mice, initially, the gene of interest will be disrupted functionally (i.e., its allele is knocked out) when the VCre gene is inserted into the initial ATG of the gene of interest. Because tissue-specific VCre-expressing mice are usually heterozygous for one allele of the gene of interest, the other intact allele can produce the gene product of interest; typically, these mice grow normally. Nonetheless, one should be aware of the potential for haploinsufficiency. In rare cases, functional differences or defects can occur

due to the different quantity of transcripts expressed by one allele versus two alleles. In such cases, the VCre gene should be inserted into the gene of interest after an internal ribosome entry site (IRES) sequence, which is inserted just after the stop codon of the CDS of one of the last exons. If it is acceptable for the gene of interest to have a few amino acids fused to its C terminus, the VCre gene should be inserted after a 2A self-cleaving sequence, which is inserted in place of the termination codon.

6. Because Cre originates from P1 phage, which can infect *E. coli*, some researchers use an artificially synthesized Cre gene (known as improved Cre or iCre) optimized for mouse and human codon usage. The iCre gene is also designed to reduce the high CpG content of the prokaryotic coding sequence, thereby reducing the chances of epigenetic silencing in mammals [26]. Because the artificially synthesized VCre and SCre genes to be used for cell cultures and mice are optimized for mouse codon usage and lacks all CpG dinucleotides, the codon does not need to be optimized again before use. Optimizing it again will not significantly improve its recombination efficiency.
7. When a VCre gene with an initial ATG is inserted into the gene of interest just before the gene's initiation codon, two ATGs result, one from the VCre gene and the other from the gene of interest. The initial ATG of the gene of interest is located at the 5′-end of the right homology arm. The question arises: Should this original ATG be deleted? Some researchers want to ensure that the gene of interest is completely knocked out in the allele containing a knock-in, because they also want to use that knock-in allele as a knockout allele of the gene of interest. So, they sometimes delete an entire exon in addition to the initial ATG. Typically, we retain the original ATG at the 5′ end of the right homology arm, because we do not know exactly which region contains transcriptional regulation signals.
8. Should a foreign polyA additional signal be added? When the VCre gene is inserted into the gene of interest just before the gene's initiation codon, the gene's polyA additional signal remains intact. When the number of exons is small and when the exons are not far from the initial ATG codon and the gene's polyA additional signal, the gene's polyA additional signal can be used. Adding a foreign polyA additional signal would not be necessary. However, especially, when the gene of interest has a lot of exons, its initial ATG codon is expected to be far from its polyA additional signal. In this case, there is no guarantee that all of its transcripts will be transcribed through its last exon. Thus, a foreign polyA additional signal should be inserted just after the VCre gene. Typically, we insert an SV40 polyA additional signal just after the VCre gene.

9. The construct must contain an expression cassette harboring an antibiotic-resistant gene, which serves as a positive selection marker. An expression cassette harboring a Neomycin-resistant gene is typically used. This Neo expression cassette contains a mouse PGK promoter, a neomycin-resistant gene, and an artificially synthesized polyA additional signal, flanked by two FRT sites. After obtaining ES cell clones that have undergone correct homologous recombination, the neo expression cassette will no longer be necessary. After chimeric or heterozygous mice are born, the mice should be crossbred with Flp-expressing mice to remove the Neo expression cassette. The Neo expression cassette should be excised so as not to hinder the natural expression pattern of the gene of interest. However, some researchers prefer to use heterozygous or homozygous mice that harbor a Neo expression cassette for further analysis. This strategy saves time, as mating with Flp-expressing mice is avoided. I strongly recommend removing the Neo expression cassette, because sometimes we have experienced problems like no expression or reduced expression of the gene of interest when not removing the Neo expression cassette.
10. The longer the homologous region, the higher the empirical homologous recombination efficiency. Generally, it is necessary to confirm that homologous recombination occurred in the correct place and not by random integration. Confirmation is typically done through PCR amplification of a PCR primer having a sequence outside of the upstream homology arm and a PCR primer having a sequence inside the Neo gene at the 5′ end of the knock-in cassette, and a PCR primer having a sequence inside of the Neo gene and a PCR primer having a sequence of outside of the downstream homology arm at the 3′ end of the knock-in cassette. For this reason, the length of the homology arms upstream and downstream (often called left arm and right arm, respectively) preferably should be around 3 kbp. If the homology arms are longer, it is difficult to amplify the PCR product using roughly purified genomic DNA from mouse ES cells. When low-quality DNA is used as a template for PCR amplification, long PCR products cannot be amplified reliably.
11. It is very important to match the DNA sequence in the homology arms to the DNA sequence in the substrain of mice from which the ES cells originated in order to increase the efficiency of homologous recombination in the ES cells. Since we typically use lab-made ES cells originating from hybrid embryos derived from C57BL/6J and C57BL/6N mice, we construct the targeting vector using a BAC clone originating from the genomic DNA of C57BL/6J or C57BL/6N mice. There are

many SNPs and differences in repeat sequences in the DNA sequence of mouse substrains. It is widely known that these DNA sequence differences significantly decreases the efficiency of homologous recombination.

12. A DT-A expression cassette can be used as a negative selection marker. The DT-A expression cassette contains a mouse PGK promoter, a diphtheria toxin A subunit gene, and an artificial polyA additional signal. Alternatively, a TK expression cassette can be used as a negative selection marker. When ganciclovir (GANC) is added to culture medium, "false" ES cells (i.e., cells with randomly integrated targeting vector containing the TK gene) will die out. There is not a big difference between the efficiency of DT-A and TK in terms of negative selection markers. Both Neo expression and DT-A expression cassettes contain a mouse PGK promoter. When the Neo expression cassette and the DT-A expression cassette are positioned in the same orientation, we notice the appearance of small deleted plasmid DNA throughout the growth of *E. coli*. The deleted plasmid DNA results from accidental homologous recombination between the two mouse PGK promoters, which are located in the same orientation. When a new targeting vector is being designed, it is very important to avoid having two of the same promoters and two polyA additional signals, especially, in the same orientation, as to avoid unexpected problems.
13. Typically, we add a restriction endonuclease NotI site (GCGGCCGC). When homologous arms contain a NotI site, we add an AsiSI site (GCGATCGC) or an isoschizomer SgfI in place of the NotI site.
14. We always confirm the DNA sequence of critical regions of a targeting vector by Sanger DNA sequencing (BigDye™ Terminator v3.1 Cycle Sequencing Kit, ThermoFisher Scientific). Especially when part of the targeting vector is amplified through PCR amplification, we always confirm the entire region produced by PCR via DNA sequencing.
15. We typically use Sequencher® software for DNA analysis. SnapGene® and ApE are also useful for analyzing DNA when designing the DNA sequence of targeting vectors.
16. After designing the targeting vector in silico, typically, we construct the targeting vector using the double Red recombination method. This procedure is used for homologous recombination in *E. coli*. BAC clones containing mouse genomic DNA originating from C57BL/6J or C57BL/6N mice are obtained from ThermoFisher or RIKEN Bioresource Center (Japan, Tsukuba). The targeting vector is constructed by carrying out Red recombination two times. First, the DNA cassette for the retrieving vector is produced by PCR amplification

using a pDT-6 plasmid containing the diphtheria toxin A (DT-A) chain. The first Red recombination is performed in order to construct the first plasmid from a BAC clone containing the gene of interest using the DNA cassette for retrieving. Second, the VCre knock-in cassette containing an SV40 polyA additional signal and FRT-Neo-FRT is produced by serial PCR amplification using a plasmid containing the VCre gene as a template. To integrate the VCre knock-in cassette just before the initial ATG of the gene of interest, we perform a second Red recombination. We always confirm the DNA sequence of the region of interest, which is produced by PCR amplification and then sequenced by Sanger DNA sequencing. A conventional ligation method can be used to construct the targeting vector. Alternatively, the targeting vector can be ordered from a DNA synthesis or outsourcing company, like VectorBuilder. Designing the targeting vector is the most important thing; its construction can be outsourced to a contractor.

17. There are two major inducible Cre systems: CreERT2, which is induced by adding tamoxifen, and the TET on/TET off system, which is induced by adding doxycycline [2]. Inducible VCre systems can be potentially very useful and powerful tools, especially if both tamoxifen-inducible Cre and doxycycline-inducible VCre are at work in the same cells of a particular mouse or in the reverse combination. Our laboratory has attempted to create tamoxifen-inducible VCre fused to ERT2 and doxycycline-inducible VCre based on the TET on system (unpublished data). One needs to confirm whether an NLS sequence is present or absent. When only VCre is used, an NLS sequence is needed to move it to the nucleus, but when VCre is fused to ERT2, the NLS sequence needs to be removed from the conventional VCre sequence if it is to remain in the cytoplasm in situations lacking tamoxifen.
18. Which should be used first—VCre or SCre? Our group has more experience with using VCre than SCre. As many researchers use VCre without complaint, we conclude that VCre is less troublesome. Our assay in *E. coli* demonstrated that the efficiency of site-specific recombination of VCre and SCre is almost the same. Using other assays, some have reported SCre to have a relatively lower efficiency than VCre [19]. For these reasons, I recommend VCre to be used first.
19. Dre/rox is another famous site-specific recombination system [27, 28]. Notably, some researchers have reported that Dre/rox and Cre/loxP cross-react with each other [19, 29]. This potential pitfall should not be overlooked.
20. Since our initial report of VCre and SCre and their recognition sites, three site-specific recombinases (SSR), Viki/vox [30],

Nigri/nox, and Panto/pox [31], have been reported using very similar screening methods previously reported by us. As Panto/pox cross-reacts with Dre/rox, it is necessary to carefully consider suitable combinations of recombinases that do not cross-react and recombination efficiency in mice when using them.

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Chapter 14

Efficient CRISPR/Cas9-Assisted Knockin of Large DNA Donors by Pronuclear Microinjection During S-Phase in Mouse Zygotes

Takaya Abe, Ken-ichi Inoue, and Hiroshi Kiyonari

Abstract

In the CRISPR/Cas9-mediated gene cassette knockin (KI) strategy, a gene cassette is integrated into a target locus through a proper DNA repair pathway after the Cas9-induced double-strand DNA breaks; the activation of the DNA repair pathway is known to be correlated with the cell cycle. Recently, we have reported a new KI approach named SPRINT (*S*-phase *pr*onuclear *in*jection for *t*argeting)-CRISPR, focusing on the correlation between the cell cycle and the KI efficiency in the mouse zygote microinjection. Our results suggest that the CRISPR-mediated KI with a homologous recombination-based donor vector during S-phase enhances the KI efficiency. For SPRINT-CRISPR, the uniformity of the zygotes in the cell cycle is achieved by in vitro fertilization, and the zygotes are cryopreserved until use. These reproductive techniques are necessary for efficient KI. Furthermore, Piezo-assisted microinjection has been successful in improving the survival rate of the injected embryos. In this chapter, we describe the protocols that focus on the zygote preparation and Piezo-assisted microinjection of the SPRINT-CRISPR method.

Key words CRISPR/Cas9, Knockin mouse, In vitro fertilization, Frozen zygotes, Piezo-assisted microinjection, Homologous recombination

1 Introduction

CRISPR/Cas9-mediated genome editing enables the one-step generation of genetically engineered mice through zygote manipulations such as microinjection and electroporation [1–5]. However, the gene cassette knockin (KI) with a large DNA donor is known to be inefficient compared to short fragment KIs such as a loxP. To overcome this issue, we have surveyed the KI method, including CRISPR components, and investigated the appropriate injection timing to improve the efficiency of KI. We have found that a relatively high-density donor vector and a crRNA-tracrRNA ribonucleoprotein (ctRNP) should be introduced into a pronucleus a few hours prior to nuclear envelope breakdown, during the S-phase

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of the pronuclear stage [6]. Thus, an efficient approach by *S*-phase *pr*onuclear *in*jection for *t*argeting the DNA KI donor in mouse zygotes was developed and named SPRINT-CRISPR.

For SPRINT-CRISPR, the pronuclear-stage zygotes produced by in vitro fertilization (IVF) are essential to make the cell cycle uniform at the S-phase, and a ctRNP is employed to activate the Cas9 protein efficiently and quickly after microinjection. We also utilized Piezo-associated microinjection to improve the survival ratio of the injected zygotes. In this chapter, we provide an instructional guide and tips for the design of this KI strategy including in vitro fertilization, freezing and thawing of pronuclear-stage zygotes, and Piezo-associated microinjection.

2 Materials

2.1 Design and Construction of the Donor Vector

1. CRISPRdirect: https://crispr.dbcls.jp [7].
2. Cloning vector: pBlueScript II SK (+).
3. Endotoxin-free plasmid DNA isolation kit.
4. High-performance liquid chromatography (HPLC)-purified crRNA and tracrRNA.

2.2 Preparation of Pronuclear-Stage Zygotes

1. C57BL/6 mice (10–16 weeks old).
2. Pregnant mare serum gonadotropin (PMSG).
3. Human chorionic gonadotropin (hCG).
4. Incubator (37 °C, 5% CO_2).
5. mHTF medium: Table 1, homemade.
6. Paraffin oil.
7. Hyaluronidase.
8. Chiller (AS ONE, EC-40RA).
9. DAP213 (2 M DMSO, 1 M acetamide, 3 M propylene glycol): Table 2, homemade [8].
10. PB1 medium: Table 3, homemade.
11. 1 M DMSO: 1 M DMSO solution in PB1 medium, Table 3, homemade.
12. mWM: Table 4, homemade.
13. 0.25 M sucrose: 0.25 M sucrose in PB1 medium, Table 3, homemade.
14. Cryotube.
15. 35 mm dish.
16. Glass capillary pipette: pulled using the flame of an alcohol lamp.

Table 1
Composition of mHTF medium

mHTF medium	*500 ml*
NaCl	2.969 g
KCl	0.175 g
$MgSO_4 \cdot 7H_2O$	0.0245 g
KH_2PO_4	0.027 g
$CaCl_2$	0.285 g
$NaHCO_3$	1.05 g
D-Glucose	0.25 g
Na-lactate	1.7 ml
Na-pyruvate	0.0185 g
Penicillin G	0.0375 g
Streptomycin	0.025 g
0.5% phenol red	0.2 ml
BSA	2 g
MilliQ water	up to 500 ml

Table 2
Composition of DAP213

Solution A	*100 ml*
Acetamide	11.814 g
PB1	up to 100 ml

DAP213	*200 ml*
Solution A	100 ml
PB1	23.088 ml
DMSO	31.252 ml
Propylene glycol	45.66 ml

2.3 Pronuclear Injection

1. Microscope (Leica, DMi8).
2. Micromanipulators (Eppendorf, TransferMan 4r).
3. Microinjector (Eppendorf, CellTram 4r).
4. Piezo driver (Eppendorf, PiezoXpert).

Table 3
Composition of PB1 medium, 0.25 M Sucrose, and 1 M DMSO

PB1 medium	*500 ml*
NaCl	4 g
KCl	0.1 g
$CaCl_2$	0.06 g
KH_2PO_4	0.1 g
$MgCl_2 \cdot 6H_2O$	0.05 g
Na_2HPO_4	0.575 g
Na-pyruvate	0.018 g
D-Glucose	0.5 g
Penicillin G	0.0375 g
BSA	1.5 g
MilliQ water	up to 500 ml

0.25 M Sucrose	*500 ml*
PB1 medium	500 ml
Sucrose	42.788 g

1 M DMSO	*200 ml*
PB1 medium	184.374 ml
DMSO	15.626 ml

5. Glass capillary.
6. Polyethylene tubing.
7. 10 mL syringe.
8. H_2SO_4 (sulfuric acid).
9. Micropipette puller (Sutter Instrument, P-1000 IVF).
10. Microforge (NARISHIGE, MF2).
11. HF (hydrofluoric acid): 55% hydrofluoric acid.
12. Hg (mercury).
13. M2 medium.
14. Nuclease-free water.
15. Cas9 protein.

Table 4
Composition of mWM medium

mWM medium	*500 ml*
NaCl	3.2 g
KCl	0.178 g
KH_2PO_4	0.081 g
$MgSO_4 \cdot 7H_2O$	0.147 g
$NaHCO_3$	0.95 g
D-Glucose	0.5 g
Na-pyruvate	0.0125 g
Ca-lactate	0.23 g
Penicillin G	0.0375 g
Streptomycin	0.025 g
20 mM 2-Mercaptoethanol	0.25 ml
100 mM EDTA-2Na	0.25 ml
0.5% phenol red	0.5 ml
BSA	1.5 g
MilliQ water	up to 500 ml

16. PVP medium: 12% PVP (polyvinylpyrrolidone, Merck, P5288) in PB1, homemade.
17. 50° silicone gasket (Kokugo), custom-made.
18. 40 mm × 50 mm glass slide (Matsunami Glass), custom-made.
19. Beeswax.
20. 1 mL syringe.
21. Foster mother mice (ICR).

3 Methods

3.1 Design and Construction of a Donor Vector

1. Choose a gRNA site as the target for insertion of a gene cassette using Web tools. A highly specific gRNA is recommended to minimize the off-target effects. The DNA double-strand break (DSB) by Cas9 nuclease generally occurs at the 3 bases 5′-upstream of the protospacer adjacent motif (PAM) sequence, where the gene cassette can be inserted most efficiently (Fig. 1) (*see* **Note 1**).
2. Synthesize a crRNA and a tracrRNA through a commercial company.

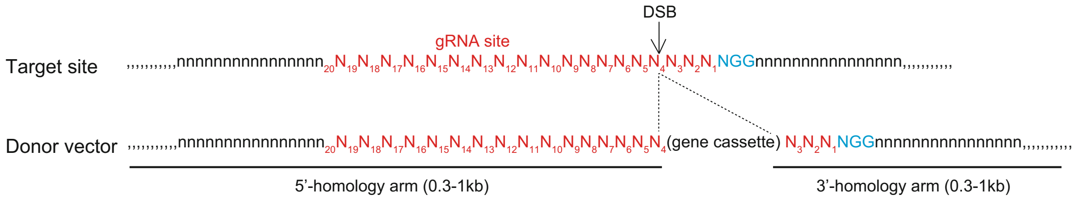
DSB
gRNA site
Target site
,,,,,,,,,,nnnnnnnnnnnnnnnn$N_{20}N_{19}N_{18}N_{17}N_{16}N_{15}N_{14}N_{13}N_{12}N_{11}N_{10}N_9N_8N_7N_6N_5N_4N_3N_2N_1$NGGnnnnnnnnnnnnnnn,,,,,,,,,,
Donor vector
,,,,,,,,,,nnnnnnnnnnnnnnnn$N_{20}N_{19}N_{18}N_{17}N_{16}N_{15}N_{14}N_{13}N_{12}N_{11}N_{10}N_9N_8N_7N_6N_5N_4$(gene cassette) $N_3N_2N_1$NGGnnnnnnnnnnnnnnn,,,,,,,,,,
5'-homology arm (0.3-1kb)
3'-homology arm (0.3-1kb)

Fig. 1 Schematic representation of a genomic target site and a donor vector

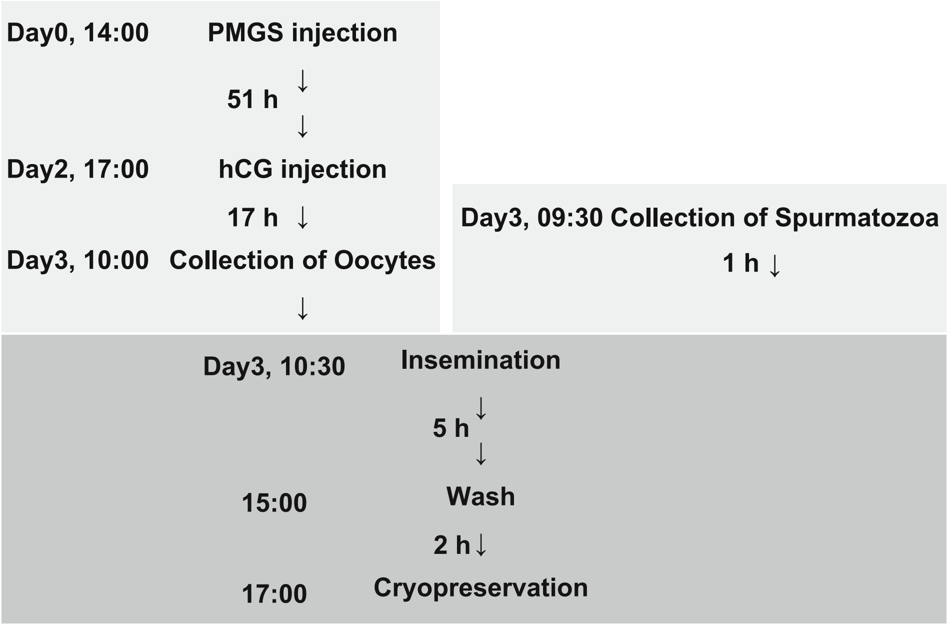

Fig. 2 A practical time schedule of in vitro fertilization and cryopreservation

3. Design homology arms (from 0.3 to 1 kbp) (*see* **Note 2**).
4. Amplify the homology arms by PCR with primers containing an appropriate restriction enzyme site for subcloning.
5. Construct a donor vector by inserting the homology arms and a cassette into a cloning vector such as pBlueScript II SK (+) using a standard cloning method.
6. Purify the donor vector using an endotoxin-free plasmid DNA isolation kit. Adjust the concentration to 2 μg/μL with Tris-EDTA (TE) buffer. Store at −20 °C until use.

3.2 Preparation of Frozen Pronuclear-Stage Zygotes

3.2.1 Superovulation

1. Administer 5 IU of pregnant mare serum gonadotropin (PMSG) by intraperitoneal injection to mature female mice (10–16 weeks old) (Fig. 2).
2. After 48–52 h, administer 5 IU of human chorionic gonadotropin (hCG) by intraperitoneal injection.

3. After 16–18 h (the day of the IVF procedure), collect oocytes (*see* **step 3** in Subheading 2.3 for details).

3.2.2 Collection of Spermatozoa

1. The evening before the IVF procedure, incubate 100 μL of an mHTF drop covered with paraffin oil on a 35 mm dish (sperm dish) (Table 1).
2. After sacrifice of the mature male mouse (aged 10–48 weeks), immediately dissect out the cauda epididymides, and place them on the filter paper, removing as much blood and fat as possible (Fig. 2).
3. Collect the fresh sperm with the dissecting needle from the cauda epididymis cut with scissors and place in the mHTF drop.
4. For capacitation, place the dish in the incubator for at least 1 h prior to insemination (by 10 a.m. at the latest).

3.2.3 Collection of Oocytes

1. The evening before performing IVF, incubate 100 μL of the mHTF drop covered with paraffin oil on a 35 mm dish (fertilization dish) (Table 1).
2. After sacrifice, immediately dissect out the oviducts of the superovulation females and place them on the filter paper to remove as much blood as possible (Fig. 2).
3. Place the oviducts in the paraffin oil part of the fertilization dish.
4. Tear the oviduct ampulla with a dissection needle and pull out the cumulus-oocyte complex (COC).
5. Transfer the COC into the mHTF drop with the dissection needle (*see* **Note 3**).
6. Incubate until insemination (*see* **Note 4**).

3.2.4 In Vitro Fertilization

1. Before insemination, place four drops of 50 μL mWM containing 0.1% hyaluronidase covered with paraffin oil on a 35 mm dish (this is the first washing dish), and place four drops of 50 μL mWM covered with paraffin oil on a 35 mm dish (this is the second washing dish) (Table 4).
2. Transfer the 1–2 μL of sperm after capacitation to the HTF drop containing the oocytes (fertilization dish) (Fig. 2) (*see* **Note 5**).
3. After 4–5 h of incubation, transfer and wash the oocytes in the fertilization dish with a glass capillary pipette using the drops in the first washing dish.
4. Pick the pronuclear-stage zygotes up from the final drop, and wash them using the drops in the second washing dish. Unfertilized and parthenogenic oocytes are removed by this step.

3.2.5 Cryopreservation

The zygotes were cryopreserved by a simple vitrification method [8]:

1. Place two drops of 50 μL DMSO (1 M) on a 35 mm dish for each cryotube (*see* **Note 6**).
2. Transfer and rinse zygotes using the DMSO (1 M) drops (*see* **Notes 7** and **8**).
3. From the second drop, pick up zygotes in 5 μL of DMSO (1 M) and transfer to the bottom of a cryotube using a pipette.
4. Let the cryotube stand for 5 min at room temperature.
5. Transfer the cryotube to a chiller set to −3 °C, and let it chill for 5 min (*see* **Note 9**).
6. Add 95 μL of DAP213 (0 °C) to the cryotube (Tables 2 and 3), while still in the chiller; do not mix by pipetting and let stand for just 5 min (*see* **Note 10**).
7. Transfer the cryotube into liquid nitrogen (LN_2) quickly but gently (*see* **Notes 11** and **12**).
8. Store the cryotube in LN_2 until use (*see* **Note 13**).

3.3 Thawing Zygotes

1. Place four drops of 50 μL of mWM on a 35 mm dish (culture dish) and incubate until equilibrated (Table 4).
2. Remove the cryotube from LN_2.
3. Remove the screw cap and immediately discard any remaining LN_2 by inverting the cryotube.
4. Let the cryotube stand at ambient temperature for 60–90 s (*see* **Notes 14** and **15**).
5. After the pre-warming period, quickly but gently add 900 μL of pre-warmed thawing medium (0.25 M sucrose). Mix by gentle pipetting several times to thaw embryos while avoiding re-freezing (*see* **Notes 16** and **17**).
6. Transfer the thawing medium with zygotes to a new 35 mm dish.
7. Collect zygotes in the first drop of the culture dish, and then rinse them with several drops of mWM.
8. Confirm the condition of the zygotes. Typically, 95% or more of the zygotes should look normal.
9. Incubate the zygotes for approximately 1 h before injection.

3.4 Pronuclear Injection

3.4.1 Production of Injection Needle

1. Pull a glass capillary with a micropipette puller (ramp, 781; heat, 760; pull, 75; velocity, 80; time, 150; pressure, 500).
2. Attach the pulled capillary to a syringe full of air, equipped with a polyethylene tube (Fig. 3a).

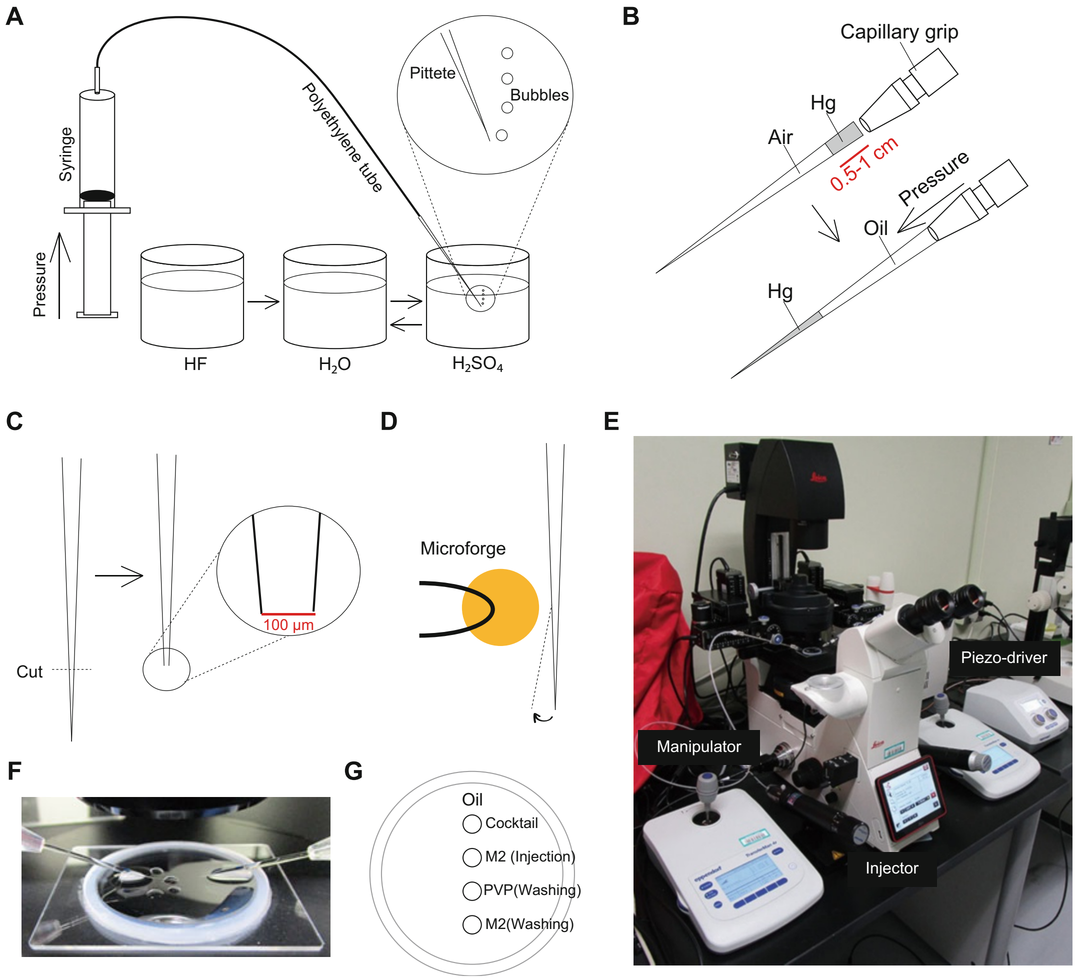

Fig. 3 Piezo-assisted microinjection setup: (**a**) making the injection needle, (**b**) attaching the injection needle, (**c**) making the holding needle, (**d**) bending the needle, (**e**) the microscope and micromanipulators with the Piezo impact driving unit, (**f**) the injection chamber, and (**g**) diagram of the injection chamber

3. Soak the tip of the capillary in approximately 25% HF for a few seconds while applying pressure with the syringe (Fig. 3a, HF) (*see* **Note 18**).
4. Rinse the capillary with water while continuing to apply pressure (Fig. 3a, H_2O).
5. Transfer the capillary to H_2SO_4 and move it rapidly from side to side while continuing to apply pressure and check for small bubbles coming out of the tip of the capillary (to determine if there is a hole) (Fig. 3a, H_2SO_4) (*see* **Note 19**).
6. Rinse the capillary with water while continuing to apply pressure (Fig. 3a, H_2O) (*see* **Note 20**).
7. Bend the tip of the capillary by heating it with the filament of a microforge (Fig. 3d) (*see* **Note 21**).

3.4.2 Production of Holding Needle

1. Pull a capillary with a micropipette puller (ramp, 764; heat, 814; pull, 30; velocity, 120; time, 200; pressure, 300).
2. Cut the tip of the pulled capillary at 100 μm diameter by scratching with an ampoule cutter to make the tip as blunt as possible (Fig. 3c).
3. Round the tip of the cut capillary with a microforge to prevent the zygote from being damaged.
4. Bend the tip of the capillary with a microforge in the same manner as the injection needle (Fig. 3d) (*see* **Note 22**).

3.4.3 Preparation of Injection Cocktail

1. Dilute crRNA (500 ng/μL) and tracrRNA (1 μg/μL) with nuclease-free water. Store at −80 °C until use.
2. Prepare 9 μL of premixture by mixing 1 μL of crRNA (500 ng/uL), 1 μL of tracrRNA (1 μg/μL), 1 μL of donor vector (100 ng/μL), and 6 μL of nuclease-free water on ice.
3. Add 1 μL of Cas9 protein (1 μg/μL) to the 9 μL of premixture, and gently mix just prior to injection. The final concentrations are as follows: crRNA, 50 ng/μL; tracrRNA, 100 ng/μL; donor vector, 10 ng/μL; and Cas9 protein, 100 ng/μL.
4. Centrifuge at 12,000 g for 15 min at 4 °C.
5. Keep on ice until use (*see* **Note 23**).

3.4.4 Prepare Injection Chamber

1. Prepare an injection chamber in advance. Attach a 50° silicone gasket to a 40 mm × 50 mm glass slide with beeswax (Fig. 3f) (*see* **Note 24**).
2. Place four drops (5 μL) on an injection chamber (Fig. 3g). The first drop contains only injection cocktail, the second is the injection drop (M2 medium containing zygotes), the third is the M2 drop, and the fourth is the PVP drop. These drops are covered with paraffin oil.
3. Set the injection chamber to the stage of a micromanipulator (Fig. 3e, f).

3.4.5 Attaching Holding Needle

1. Attach the holding needle to a microinjector to keep out air (*see* **Note 25**).
2. Move the tip of the holding needle into the M2 drop and draw up a small amount of M2 medium.

3.4.6 Attaching Injection Needle for the Piezo-Assisted Microinjection

1. Set up a micromanipulation system equipped with a Piezo driver (Fig. 3e).
2. Fill the end of the injection needle (0.5–1 mm) with Hg using a 1 mm syringe and attach it to a microinjector (Fig. 3b).
3. Push the Hg to the tip of the needle by applying pressure with the microinjector.

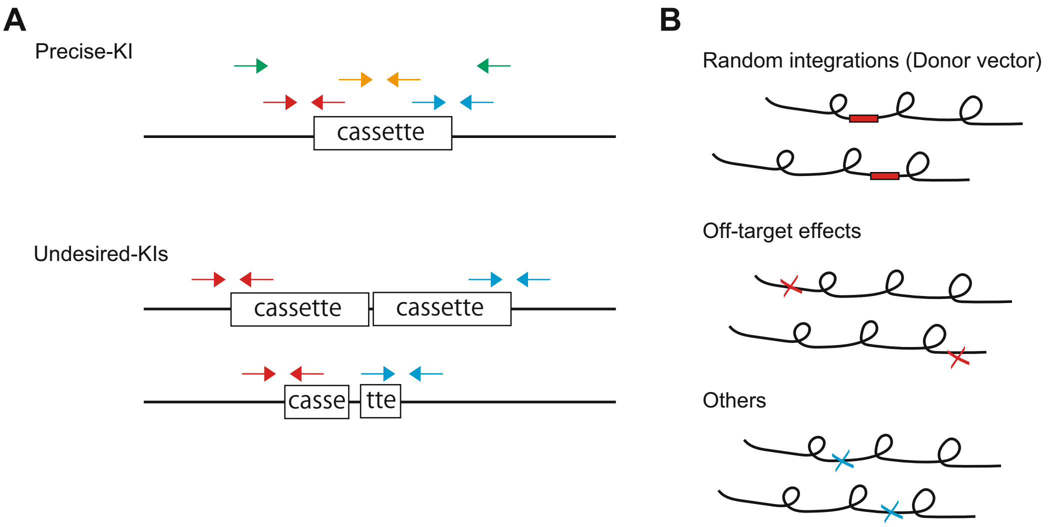

Fig. 4 Diagram of genetic modification with genome editing: (**a**) target site and (**b**) non-target site. Arrows indicate PCR primers

4. While activating the Piezo, rinse the injection needle once with PVP medium (*see* **Note 26**).
5. Move the tip of the needle into the M2 drop and wash the needle with M2 medium to remove the residue PVP.
6. Move the tip of the needle into the injection drop and fill with the injection cocktail.

3.4.7 Piezo-Assisted Microinjection

1. Transfer zygotes into the injection drop. The transferred zygotes should be injected within 30 min (*see* **Note 27**).
2. Move both the injection and holding needles into the injection drop.
3. Hold a zygote with the holding needle and inject the cocktail into a paternal pronucleus (*see* **Notes 28** and **29**).
4. Incubate the injected zygotes for 1 h with mWM medium and transfer into the ampulla of pseudopregnant females (0.5-day post-coitus).

3.5 Genotyping

1. Design PCR primers for detection of a KI allele and random integrations (Fig. 4a, b) (*see* **Note 30**).
2. Collect the tissue of the F0 mice in a 0.6 mL tube and add 180 μL of 50 mM NaOH.
3. Heat at 95 °C for 10 min.
4. Add 20 μL of 1 M Tris–HCl (pH 8.0).
5. Vortex and centrifuge at 12,000 g for 10 min.
6. Use 2 μL of the extract for 25 μL PCR reaction solution.

7. Analyze the PCR products by electrophoresis.
8. The genomic DNAs of the PCR-positive mouse should be further purified and analyzed by sequencing (*see* **Note 31**).

4 Notes

1. A KI cassette can be inserted anywhere within the gRNA target sequences, but KI efficiency may decrease as the distance from the DSB site increases.
2. Micro-homology arms for the MMEJ (microhomology-mediated end joining)-based method are 40 bp [9].
3. Up to four female COCs can be added per fertilization dish.
4. The number of mice that can be dissected at one time depends on the number for which the above procedure can be completed within 10 min.
5. The sperm suspension (1 μL) will contain 400–800 sperm.
6. The DMSO drops are not covered with paraffin oil.
7. The number of zygotes in a cryotube depends on the number for which the zygote injection can be performed at one session.
8. Before transferring the zygotes to the second drop, make sure they have sunk to the bottom of the first drop.
9. The temperature of the chiller is set so that the temperature inside the cryotube is 0 °C.
10. Do not let the mixture stand for longer than 5 min.
11. To prevent moving the zygotes from the bottom of the cryotube until frozen, do not tilt the cryotube, and soak only the bottom of the cryotube in LN_2 in an upright position.
12. Overtightening the screw cap will make it difficult to remove the cap quickly when thawing.
13. The cryopreservation should be completed within 7 h after insemination.
14. Carefully monitor the change in appearance of the frozen media. Initially it will be clear—when a frost-like white ring is formed on the surface, proceed to **step 5** immediately.
15. This pre-warming period tends to depend on the temperature of the room and the type of cryotube used. The optimal duration should be empirically determined.
16. While initially adding the thawing medium, dispense only approximately two-thirds into the cryotube. Then start gentle and partial pipetting (without full strokes) to avoid formation of air bubbles and excess turbulence, which damage the embryos.

17. This step should not take longer than 2 min.
18. Do not soak the capillary in the HF for too long. Otherwise, the diameter of the hole in the capillary may become too large.
19. This step also cleans the tip of the capillary.
20. If you do not see the bubbles in **step 5**, repeat the steps, starting from **step 3** (this may need to be done 2–5 times).
21. The bending angle of the capillary depends on the setting of the micromanipulator. The needle should be parallel to the surface of the injection chamber.
22. The holding needle should be parallel to the surface of the injection chamber.
23. The injection cocktail does not need to be incubated.
24. This chamber is custom-made and can be replaced with the one usually used for microinjection.
25. Air in the tube and needle make it difficult to control the handling of zygotes.
26. This step helps maintain the potency of the Piezo.
27. The number of zygotes in the drop depends on the number on which microinjection can be performed within 30 min.
28. The Piezo driver is activated at the point of penetrating a zona pellucida and a nuclear membrane.
29. Both paternal and maternal pronuclear injections can generate biallelic KI mice [6].
30. The inserted gene should be identified by PCR using primer pairs (indicated in Fig. 4a) to distinguish between a precise KI allele and undesired KI alleles. In addition, the off-target candidate sites are identified as needed.
31. F0 KI mice may carry undesired mutations caused by random integration, off-target effects, etc. (Fig. 4b). To segregate these mutations, mate with wild-type mice for backcross as needed.

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Chapter 15

Genome Editing of Murine Liver Hepatocytes by AAV Vector-Mediated Expression of Cas9 In Vivo

Yuji Kashiwakura and Tsukasa Ohmori

Abstract

Adeno-associated virus (AAV) vectors are attractive tools for gene transfer to the liver and are used as gene therapeutic drugs for inherited disorders. The intravenous injection of an AAV vector harboring the gene of interest driven by the hepatocyte-specific promoter could efficiently express the target gene in liver hepatocytes. The delivery of genome editing tools including Cas9 and gRNA, by the AAV vector, can efficiently disrupt the target gene expression in the liver in vivo by intravenous administration in mice. We can quickly obtain mice lacking specific gene expression in the liver only by administering the AAV vector. The method could be suitable for developing genome editing treatments for inherited disorders and basic research exploring the physiological role of the target gene produced from liver hepatocytes.

Key words Adeno-associated virus vector, CRISPR-Cas9, Hepatocyte-specific gene expression, Intravenous administration, Mouse model

1 Introduction

Adeno-associated virus (AAV) vectors have attracted gene delivery systems for gene therapy to treat various hereditary diseases [1]. AAV is identified initially as a small virus-like particle associated with adenovirus [2]. AAV is a small single-strand DNA virus with Rep/Cap sequence flanked by two hairpin sequences called internal terminal repeats (ITR) (*see* Fig. 1a) [3]. Various AAV serotypes exist and have different organ tropisms [4]. AAV can efficiently infect quiescent cells, including liver hepatocytes and the central nervous system in vivo.

Additionally, AAV is unrelated to human pathology and is less immunogenic than adenovirus [5]. AAV-derived genes are unlikely to be integrated into chromosomal DNA and instead exist as concatemers in episomes of the nucleus. Hence, AAV vectors are a safe and efficient approach for delivering ectopic genes in vivo. One of the critical drawbacks of the AAV vector is that its packaging

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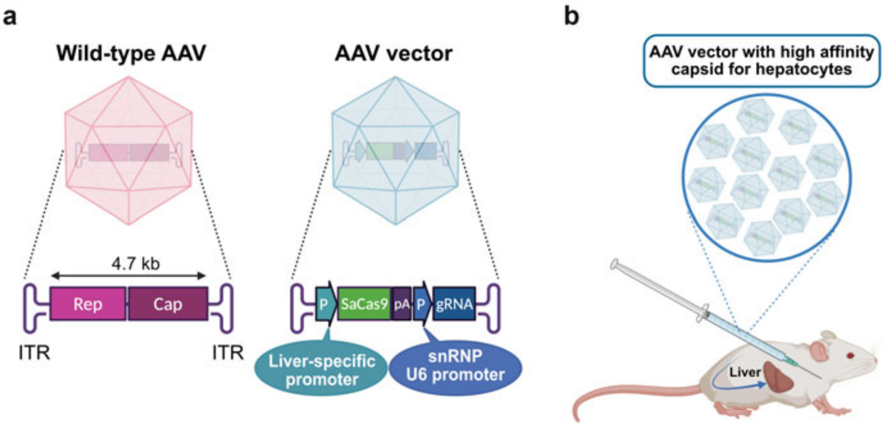

Fig. 1 Gene transfer with AAV vectors. (**a**) Structure of the AAV vector: Wild-type AAV is a single-stranded DNA virus encoding the Rep and Cap genes. Genome editing using the AAV vector uses the recombinant AAV vector with the SaCas9 gene downstream of the liver-specific promoter and gRNA driven by U6 promoter instead of the Rep/Cap gene. (**b**) Liver-targeted genome editing with AAV vectors: the use of liver-directed AAV vectors enables the introduction of the Cas9 gene into the liver by intravenous administration

capacity is limited to approximately 5 kb [6]. Therefore, we cannot accommodate large genes in an AAV vector, including *Streptococcus pyogenes* Cas9 (SpCas9) (4107 bp).

AAV vectors can efficiently transduce a target gene into the liver by intravenous injection (*see* Fig. 1b). They can be used to develop gene therapies to treat inherited diseases and for basic research to modify the target gene of the liver in animals in vivo. *Staphylococcus aureus* Cas9 (SaCas9) (3156 bp), a smaller Cas9, has been identified and can be equipped in one AAV vector [7]. AAV-mediated expression of SaCas9 and gRNA targeting *Pcsk9* could efficiently reduce PCSK9 plasma levels [8]. We have also reported that the AAV vector harboring SaCas9 and gRNA could disrupt the target gene, thereby reducing the plasma protein (coagulation factor IX, FIX; antithrombin, AT) produced by liver hepatocytes [9]. Plasma levels of FIX in mice treated by AAV-mediated gene disruption following SaCas9 expression reached those of FIX-deficient mice created by gene targeting [9]. We can easily disrupt a target gene in liver hepatocytes only by the intravenous injection of the AAV vector and obtain a disease model of liver diseases. Here we introduce the methods to disrupt the target gene in the mouse liver by AAV vector-medicated expression of Cas9.

2 Materials

2.1 Plasmids

1. An AAV plasmid harboring SaCas9 cDNA and gRNA for the target gene between ITRs (*see* Fig. 2 and **Notes 1–3**).

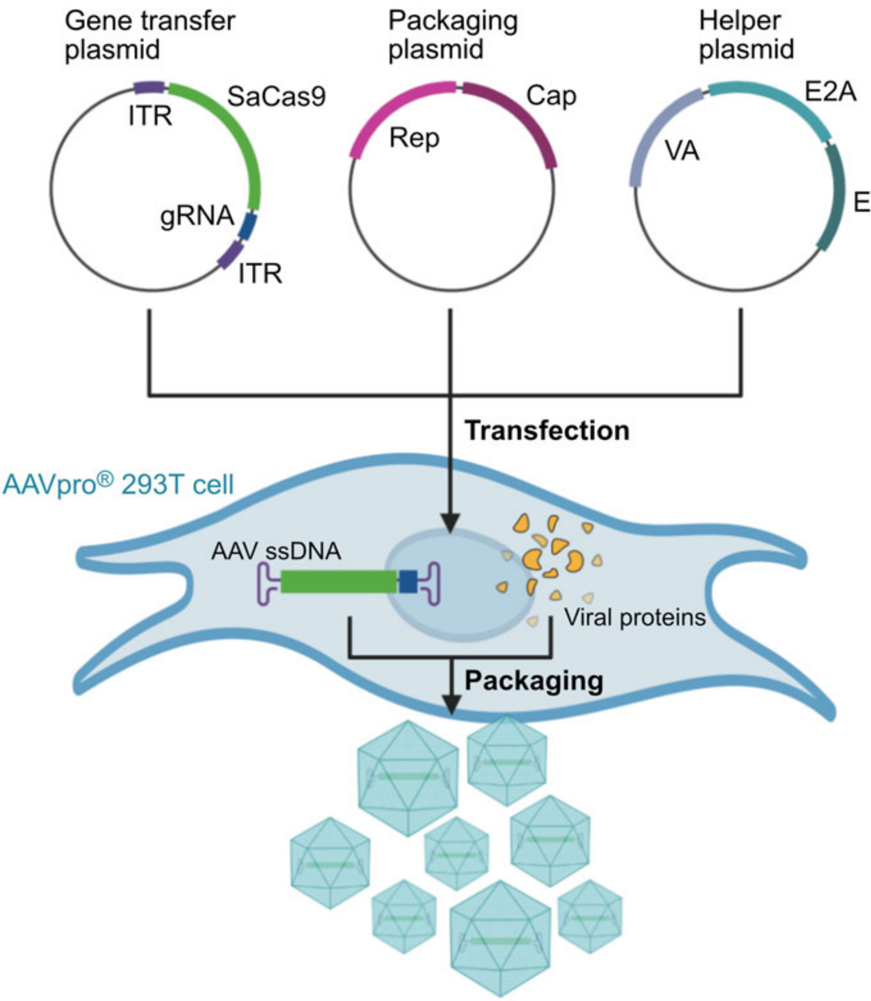

Fig. 2 AAV vector production system. Helper-free system to produce AAV vectors refers to the production system without helper viruses, including adenoviruses or herpesviruses. The gene transfer plasmid, the packaging plasmid encoding Rep/Cap gene, and the helper plasmid containing adenovirus gene products for virus replication are simultaneously transfected into AAVpro® 293 cells. The AAV vector can then be produced from the cells. (Modified from Ohmori [12])

2. A helper plasmid expressing genes (E2A, E4, and VA) derived from adenovirus (*see* Fig. 2 and **Note 3**).
3. A Rep/Cap expression plasmid (*see* Fig. 2 and **Note 3**).
4. Plasmid purification kit such as QIAprep Spin Miniprep Kit (QIAGEN).
5. Plasmid purification kit such as QIAGEN Plasmid Maxi Kit (QIAGEN).
6. Restriction enzymes which recognizes the 5′GGTCTC(N1)/(N5)3′ sequence such as BsaI, Bso31I, BspTNI, or Eco31I.

7. Buffer solution required for enzyme reaction such as CutSmart Buffer (New England BioLabs).
8. Calf intestine alkaline phosphatase.
9. Agarose gel and electrophoresis system.
10. DNA extraction kit from agarose gel such as MinElute Gel Extraction Kit (QIAGEN).
11. Sense and antisense oligo for gRNA.
12. T4 polynucleotide kinase.
13. Ultrapure water.
14. DNA ligation kit for plasmid construction such as DNA Ligation Kit Ver. 2.1 (Takara Bio).
15. *Escherichia coli* competent cells.
16. SOC medium: 2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.
17. LB agar plates and LB medium: 1% Bacto Tryptone, 0.5% yeast extract, and 1% NaCl.

2.2 Cell Culture of Murine Hepatocyte Cell Line and Plasmid Transfection

1. Murine hepatocyte cell line such as TLR3 cell line (JCRB Cell Bank).
2. DMEM/F12 with 2% FBS, L-glutamine, penicillin/streptomycin, 10 ng/mL EGF, 5 μL/L monoethanolamine, 10 μg/mL transferrin, and 1 μg/mL insulin.
3. 0.25% trypsin/EDTA.
4. Serum-free medium such as Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific).
5. Transfection reagent such as Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific).
6. Type I collagen such as Cellmatrix Type I-C (Nitta Gelatin) (*see* **Note 4**).
7. 60 mm culture dish (*see* **Note 5**).
8. 24 well culture plate (*see* **Note 5**).

2.3 DNA Isolation

1. DNA isolation kit such as DNeasy Blood & Tissue Kits (QIAGEN) (*see* **Note 6**).

2.4 Confirmation of Genome Editing Efficiency

1. T7 endonuclease (*see* **Note 7**).
2. DNA polymerase.
3. Set of primers designed to include gRNA recognition sites (*see* **Note 8**).
4. Agarose gel and electrophoresis system or microchip electrophoresis.

2.5 Cell Culture for the Production of AAV Vector

1. HEK293 cell line with high gene transfer efficiency such as AAVpro® 293T Cell Line (Takara Bio) (*see* **Note 9**).
2. DMEM high glucose with 10% FBS, L-glutamine, and penicillin/streptomycin.
3. 0.05% trypsin/EDTA.
4. T225 culture flask.

2.6 Transfection for AAV Vector Production

1. A solution of 1 mg/mL PEI such as PEI MAX® (Polyscience), dissolved with distilled water, adjusted at pH 7.0, and filtered through a 0.22 μm filter (*see* **Note 10**).

2.7 Purification of AAV Vectors

1. AAV vector purification kit such as AAVpro® Purification Kit Maxi (Takara Bio) (*see* **Note 11**).
2. 0.5 M EDTA (pH 8.0).
3. 0.45 μm filter (included in AAVpro® Purification Kit Maxi).
4. Amicon Ultra-15 (100 kDa) filter unit (included in AAVpro® Purification Kit Maxi).
5. Amphiphilic and nonionic compounds such as Pluronic™ F68 Non-ionic Surfactant (Thermo Fisher Scientific).

2.8 AAV Titration by qPCR

1. 10× PCR buffer such as GeneAmp™ 10× PCR Buffer I (Thermo Fisher Scientific).
2. Amphiphilic and nonionic compounds such as Pluronic™ F68 Non-ionic Surfactant (Thermo Fisher Scientific).
3. Sheared salmon sperm DNA (200 μg/mL).
4. Nuclease-free water.
5. Sample dilution buffer: 1× PCR buffer, 2 μg/mL sheared salmon sperm DNA, and 0.05% Pluronic™ F68 in nuclease-free water.
6. Linearized DNA cleanup kit such as QIAquick PCR purification kit (QIAGEN).
7. DNase- and RNase-free 1.5 mL tube.
8. DNase I (10 U/mL).
9. DNase digestion buffer: 13 mM Tris–HCl, 5 mM $MgCl_2$, 0.12 mM $CaCl_2$, and pH 7.5.
10. 0.5 M EDTA.
11. AAV Reference Standard Material and AAV2 or AAV8 from ATCC (*see* **Note 12**).
12. SV40pA Forward primer (100 μM): 5′-AGC AAT AGC ATC ACA AAT TTC ACA A-3′ (*see* **Note 13**).
13. SV40pA Reverse primer (100 μM): 5′-CCA GAC ATG ATA AGA TAC ATT GAT GAG TT-3′ (*see* **Note 13**).

14. SV40pA MGB probe (50 μM): 5′-FAM-AGC ATT TTT TTC ACT GCA TTC TAG TTG TGG TTT GTC-MGB-NFQ-3′ (*see* **Note 13**).
15. 20× probe/primer Mix: 18 μM SV40pA Forward primer, 18 μM SV40pA Reverse primer, and 5 μM SV40pA MGB probe.
16. qPCR Mastermix such as THUNDERBIRD® Probe qPCR Mix (TOYOBO).
17. Optical 96-well reaction plate and optical caps or optical plate sealer sheet for qPCR.
18. Quantitative PCR instruments.

2.9 Intravenous Vector Injection into Mice

1. C57BL/6 mice, gene-deficient mice, etc., according to the purpose (*see* **Note 14**).
2. Inhalation anesthetic such as ISOFLURANE Inhalation Solution (Pfizer).
3. Inhalation anesthesia machine for small animals such as NARCOBIT-E (Natsume Seisakusho).
4. 29G insulin syringe.
5. Surgical instruments for small animals (*see* **Note 15**).
6. Surgical suture needle.
7. Alcohol swabs (cut cotton soaked with 70% ethanol).
8. Cotton swab for hemostasis.
9. HN buffer: 50 mM HEPES and 150 mM NaCl, filtered through a 0.22 μm filter.
10. Amphiphilic and nonionic compounds such as Pluronic™ F68 non-ionic surfactant (Thermo Fisher Scientific).

2.10 Evaluation After Gene Transfer in the Mouse Liver

1. Anticoagulants for blood (*see* **Note 16**): 3.8% sodium citrate (pH 7.0–8.5), unfractionated heparin, or EDTA.
2. 29G insulin syringe.
3. Alcohol swabs (cut cotton containing 70% ethanol).
4. Cotton swab for hemostasis.
5. ELISA kits or measuring instrument for the detection of target protein.
6. Surgical instruments for small animals.
7. PBS or saline for organ perfusion.
8. DNA isolation kit such as DNeasy Blood & Tissue Kit (QIAGEN).
9. T7 endonuclease.

10. DNA polymerase such as TaKaRa ExTaq Hot Start Version (Takara Bio).
11. Set of primers designed to include gRNA recognition sites.
12. Agarose gel and electrophoresis system or microchip electrophoresis.

3 Methods

3.1 Construction of Plasmids with gRNA

1. Design gRNA by a web design tool such as "Benchling" (https://www.benchling.com). We usually design 3–5 candidate guide sequences. SaCas9 requires a gRNA sequence containing 21 bases and the "NNGRR(T)" PAM sequence in host genome (*see* Fig. 3a and **Note 17**).
2. To insert the gRNA into the BsaI site of the plasmid vector, incorporate 5′-CACCG for the sense sequence and 5′-AAAC and 3′-C for the antisense sequence (*see* Fig. 3a). The designed sense and the antisense sequences are prepared as oligo-DNA.

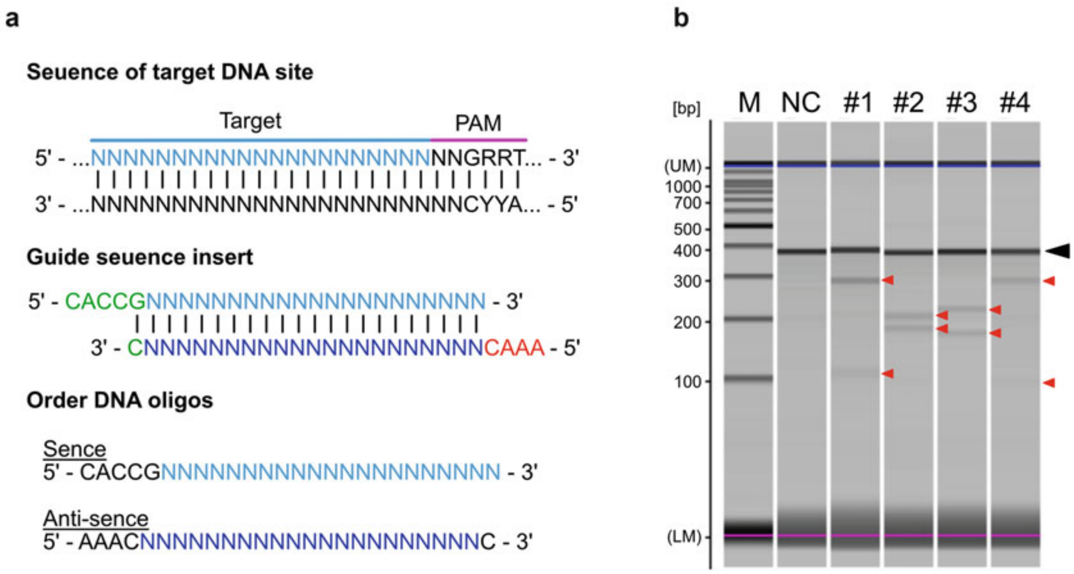
a
Seuence of target DNA site
Target
PAM
5' - ...NNNNNNNNNNNNNNNNNNNNNNNNGRRT... - 3'
3' - ...NNNNNNNNNNNNNNNNNNNNNNNNCYYA... - 5'
Guide seuence insert
5' - CACCGNNNNNNNNNNNNNNNNNNNNN - 3'
3' - CNNNNNNNNNNNNNNNNNNNNNCAAA - 5'
Order DNA oligos
Sence
5' - CACCGNNNNNNNNNNNNNNNNNNNNN - 3'
Anti-sence
5' - AAACNNNNNNNNNNNNNNNNNNNNNC - 3'
b
[bp]
M
NC
#1
#2
#3
#4
(UM)
1000
700
500
400
300
200
100
(LM)

Fig. 3 Design of guide sequence oligos and evaluation of the genome editing efficiency. (**a**) Method to design gRNA for SaCas9. The target gRNA sequence (21 nt) should be designed upstream of the PAM sequence [NNGRR(T)]. Additional sequences are incorporated into the oligonucleotide to insert the sequence into the BsaI site of the plasmid: CACCG at 5′ for sense oligonucleotide and AAAC at 5′ and C at 3′ for antisense oligonucleotide. (**b**) Example of the T7 endonuclease assay using genomic DNA from TLR3 cells transfected with the plasmid harboring SaCas9 and gRNAs. Microchip electrophoresis was used to analyze the PCR products treated with T7 endonuclease DNA fragments. Black and red arrows indicate parental PCR fragments and cleaved DNA fragments, respectively. M marker, NC negative control, #1–4 gRNA #1–4

3. Digest and dephosphorylate 1 μg of the gene transfer plasmid. For example, 1 μg plasmid is treated with 1 μL of BsaI-HF and 2 μL of CutSmart Buffer in 20 μL solution for 1 h, followed by the incubation with 0.2 U of alkaline phosphatase (calf intestine) for another 15 min.
4. Agarose gel electrophoresis of the digested plasmid.
5. Visualize DNA in the gel with DNA staining dye.
6. Confirm the length of the plasmid by visualization and then purify the plasmid band using DNA gel extraction kit.
7. Prepare annealed oligos to insert gRNA sequence. Mix each of 1 μL sense oligo (100 μM) and antisense oligo (100 μM), 1 μL 10× T4 PNK Buffer, 1 μL T4 PNK (10 U), 1 μL of 1 mM ATP, and 5 μL of ultrapure water. Anneal in a thermal cycler using the following parameters, 37 °C 2 min and 95 °C 5 min, and then ramp down to 25 °C at 5 °C/min. The annealed oligos are diluted 200 times with ultrapure water just before the ligation.
8. Ligation of the plasmid with the annealed oligos. The digested plasmid (50 ng) (**step 6**) and 1 μL of the diluted oligos (**step 7**) are ligated with DNA ligation kit (*see* **Note 18**).
9. Transform *Escherichia coli* competent cells such as DH5α with the ligated plasmid. Chill sterile polypropylene tubes on ice. Frozen competent cells are thawed on ice, and then 100 μL of the competent cells are transferred to the prechilled polypropylene tubes. Add 10 μL of **step 8** solution and incubate on ice for 30 min. Heat shock the cells for 45 s in a water bath at 42 °C. Immediately return the tubes on ice for 2 min. Add 900 μL SOC medium to competent cells, and incubate for 60 min at 37 °C with shaking. Spread the transformed competent cells (1/100 or 1/10 volume) on antibiotic LB agar plates, and then incubate at 37 °C overnight.
10. Pick a single colony from the plate and inoculate 3 mL of LB medium containing the selective antibiotic.
11. Plasmid derived from a single colony is isolated from the cultured *E. coli*.
12. Confirm the plasmid by the diagnostic digestion with the restriction enzyme and the gRNA sequence insertion by sequencing.
13. After confirming the sequence, the highly purified plasmid required for the vector production is isolated using a plasmid DNA extraction Maxi kit.

3.2 Plasmid Transfection of Murine Hepatocyte Cell Line

1. TLR3 cells are cultured in DMEM/F12 medium (Subheading 2.2, **item 2**). The cells are seeded in 60 mm collagen-coated dishes at 2 × 10^5 cells/mL and cultured in an incubator with

5% CO_2 at 33 °C (*see* **Note 19**). For cell passage, the cells are washed with PBS and then detached using 0.25% trypsin/EDTA.

2. The day before transfection, 1–2 × 10^5 cells/well of TLR3 cells are seeded on collagen-coated 24-well plates.
3. The plasmid is transfected to the cells by lipofection such as Lipofectamine 3000 according to manufacturer's instructions (*see* **Note 20**).
4. The cells are harvested 48–72 h after transfection, and genomic DNA is extracted.

3.3 T7 Endonuclease Assay

1. The T7 endonuclease assay is performed to for genome editing with DSB at the target site. The target gene fragment is amplified by PCR using genomic DNA as a template (10–50 ng).
2. Purify the PCR product by a magnetic beads such as Sera-Mag Select according to the manufacturer's recommendations.
3. The purified PCR product (18 μL) is suspended with 2 μL of NEB2 buffer (10×).
4. The PCR product is incubated at 95 °C for 5 min, 95–85 °C at −2 °C/s, and 85–25 °C at −0.1/s, and held at 4 °C.
5. The annealed PCR product (9 μL) is treated with T7 endonuclease (0.5 μL) and NEB2 buffer 2 (2×) (0.5 μL) at 37 °C for 15 min.
6. The reaction is stopped following the addition of 0.5 M EDTA (1 μL).
7. The T7 endonuclease-cleaved DNA mismatch is visualized with an agarose gel electrophoresis system or microchip electrophoresis (*see* Fig. 3b).

3.4 Production of AAV Vectors

1. AAVpro® 293T cells are maintained in DMEM medium (Subheading 2.5, **item 2**) in an incubator with 5% CO_2 at 37 °C. For cell passage, the cells are washed with PBS and then detached using 0.05% trypsin/EDTA.
2. AAVpro® 293T cells (4 × 10^6 cells) are seeded in a T225 flask four days before the transfection and monitored for 70–80% confluence on the day of transfection.
3. Perform transfection of AAVpro® 293T cells with three plasmids. Three plasmids (23 μg of each) are diluted with 3 mL DMEM in a 15 mL sterile polypropylene tube for one T225 flask (*see* **Note 21**).
4. The DMEM containing the three plasmids is mixed with 138 μL of PEI MAX solution and allows to stand at room temperature for 15 min.

5. The plasmid solution is inoculated into pre-warmed 27 mL DMEM.
6. The medium of AAVpro® 293T cells in the T225 flask is replaced with the above solution.
7. Incubate at 37 °C for 5 h.
8. Add pre-warmed 15 mL DMEM containing 900 μL of FBS (the final concentration is 2%).
9. The cells are suspended with the addition of 600 μL 0.5 M EDTA to the cultures (at room temperature for 10 min) 3 days after transfection.
10. Centrifuge the cell suspensions (2000 × *g* for 10 min at 4 °C) and discard the supernatant.
11. Centrifuge again at 2000 × *g* for 1 min at 4 °C and remove the supernatant completely (*see* **Note 22**).
12. Purify the AAV vectors from AAV purification kit. The following method is an example of extraction of AAV vector from the cells derived from one T225 flask using AAVpro® Purification Kit Maxi.
13. Loosen the cell pellets well by tapping or vortexing until no clumps of cells remain.
14. Add 2 mL of AAV Extraction Solution A plus. Resuspend thoroughly by vortexing until no clumps of cells remain.
15. Incubate for 5 min at room temperature and then vortex again for 15 s.
16. Centrifuge at 9000 × *g* for 10 min at 4 °C. Collect supernatant in a new sterile centrifuge tube using a pipette.
17. Add 1/10 volume of AAV extraction solution B to the supernatant.
18. Add 1/100 volume of Cryonase Cold-active Nuclease to the solution and then incubate at 37 °C for 1 h.
19. Add 1/10 volume of Precipitator A, vortex for 10 s, incubate at 37 °C for 30 min, and vortex again for 10 s.
20. Add 1/20 volume of Precipitator B, promptly vortex for 10 s, and then centrifuge at 9000 × *g* for 5 min at 4 °C.
21. The supernatant is collected and filtered using a 0.45 μm filter.
22. The filtrate containing AAV vector is applied into an Amicon Ultra-15 (100 kDa) filter unit. Repeat centrifugation at 2000 × *g* for 5 min at room temperature until the volume of the solution in the filter is less than 300 μL.
23. After removing the flow-through, add 1 mL of Suspension Buffer including 0.001% Pluronic F68 inside the cup of the Amicon Ultra-15 filter unit, and mix the solution uniformly by

pipetting. Repeat centrifugation at 2000 × *g* for 5 min at room temperature until the AAV solution in the filter is less than 300 μL.

24. Repeat the above washing step four times (total five times) to completely substitute the solution.
25. After discarding the flow-through, resuspend the solution inside of the cup of the Amicon Ultra-15 filter unit by vortexing for 30 s and transfer the solution to a new tube.
26. The AAV vector titer is analyzed using the method in Subheading 3.5 or by quantitative PCR such as an AAVpro® Titration Kit.
27. Store the AAV vector at −80 °C (*see* **Notes 23** and **24**).

3.5 AAV Titration by qPCR

1. Serial dilution of linearized plasmids (containing SV40 polyA) are used to generate the standard curve for AAV titration (*see* **Note 25**). Linearize the 10 μg gene transfer plasmid harboring SV40 polyA with enzyme (unique enzyme that cuts outside SV40 polyA) in the appropriate reaction buffer, preferable overnight at 37 °C. For example, 10 μg plasmid is treated with 10 U restriction enzyme and 5 μL of 10× enzyme buffer in 50 μL solution for 16 h.
2. Use 1% of digested reaction to check linearization of plasmid for agarose gel electrophoresis.
3. Clean up the linearized plasmid such as Qiaquick PCR purification Kit or appropriate purification kit. Elute with 50 μL nuclease-free water in DNase- and RNase-free 1.5 mL tube.
4. Determine the concentration and copy number of the linearized plasmid and adjust the 10^9 copy dilution with sample dilution buffer (*see* **Note 26**).
5. Dilute serially 10^8–10^2 copies of the plasmid tenfold in sample dilution buffer.
6. Digest plasmid DNA in the AAV vector with DNase I. For example, 10 μL vector sample is treated with 5 U DNase I (1 μL) and 2.5 μL of 10× DNase I buffer in a 25 μL solution at 37 °C for 1 h.
7. For enzyme inactivation, 1.25 μL of 0.5 M EDTA is added and incubated at 80 °C for 2 min, and the reaction volume is increased to 50 μL with distilled water (×5 dilution sample).
8. For the reference standard material of AAV2 or AAV8, adjust any dilution factor with sample dilution buffer (e.g., $\times 10^2$–$\times 10^7$ dilution).
9. Prepare the PCR reaction. Prepare enough mix for two reactions of each DNA sample, a standard curve, two control samples, and two reactions for no template control (H_2O).

Adjust 5 μL of each sample or standard solution, 10 μL of qPCR mastermix, 1 μL of 20× probe/primer mix, and 4 μL of distilled water (20 μL total) in one well of a 96-well plate for qPCR analysis.

10. Place the optical caps or optical plate sealer sheet over the 96-well plate. Centrifuge briefly before placing on the machine.
11. Set the conditions for 1 min at 95 °C, 40 cycles of 15 s at 95 °C, and 30 s at 60 °C, and start the PCR reaction.
12. Determine number of copies per PCR reaction for each dilution of test article using the standard curve (*see* **Note 27**). The AAV reference standard material is used to check the consistency of the obtained titers with the previously obtained data.

3.6 Intravenous Injection and Blood Drawing of the Mouse Through the Jugular Vein

1. Anesthesia is induced with 3% isoflurane and then maintained with 1–2% isoflurane. The concentration of isoflurane is adjusted to monitor the respiratory status. The surgical tools are then prepared (*see* Fig. 4a).
2. The anesthetized mouse is placed on its back, and both paws are secured with a kite string (*see* Fig. 4b).

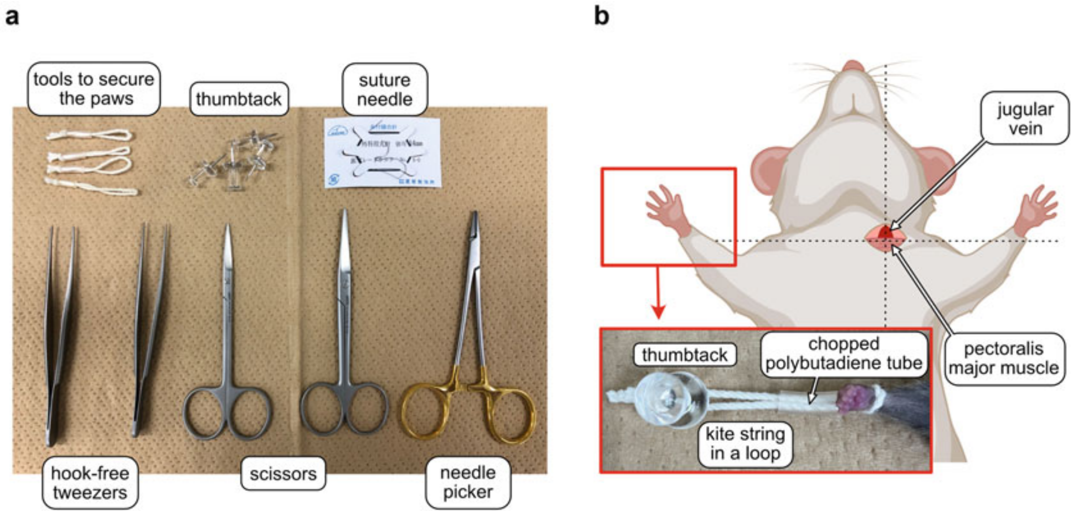

Fig. 4 How to approach the mouse jugular vein. (**a**) Examples of equipment used are suture needles, hook-free tweezers, scissors, thumbtacks, and needle pickers. The kite string that holds the mouse's paw is homemade. Distinguish scissors for skin incision from those for thread cutting. (**b**) Approach to the jugular vein of a mouse. Hold the mouse on its back. Gently hold both paws with the kite string shown in the figure below and lightly apply tension to the skin. The crossed dotted lines indicate the front leg and the external jugular lines. After disinfecting the incision site, a longitudinal incision of about 5 mm is made in the oval. The pectoralis major muscle is identified, and the upper connective tissue is peeled off with hook-free tweezers. Once the venous plexus is observed, approach the venous plexus via the pectoralis major muscle

3. Fill a syringe with a vector solution (200 μL) for vector injection or anticoagulant (*see* **Notes 28** and **29**).
4. Sterilize the skin with alcohol swabs and make a 3–5 mm skin incision with small surgical scissors. Then, a skin incision is made at a line connecting the two front legs and on the external jugular line (*see* Fig. 4b).
5. The subcutaneous connective tissue is carefully peeled off with hook-free tweezers until the jugular vein is observed above the pectoralis major muscle (*see* **Note 30**).
6. The vector or anticoagulant-containing syringe needle is inserted into the jugular vein via the pectoralis major muscle. *Do not* directly approach the jugular vein.
7. The vector solution is administered slowly after confirmation of the reversal of blood into the syringe. To draw blood, pull the plunger slowly to avoid hemolysis.
8. Remove the puncture needle after vector administration. The puncture site is compressed with a sterile cotton swab for hemostasis.
9. Close the skin incision with a thread suture needle.
10. Keep mouse at 37 °C until awakening from anesthesia.

3.7 Confirmation of Genome Editing Efficiency in the Mouse Liver

1. Evaluate the expression of proteins produced from the liver by plasma protein concentration (coagulation factor activity or ELISA) or immunohistochemistry.
2. Evaluate genome editing efficiency in the mouse liver. Mice are sacrificed by deep isoflurane anesthesia, perfused with 30 mL PBS or saline, and tissues are harvested for T7 endonuclease assay (*see* Subheading 3.3) or next-generation sequencing.

4 Notes

1. A plasmid harboring SaCas9 driven by the CMV promoter for the production can be obtained from the Addgene (#61591). gRNA sequence is directly inserted into the BsaI site. The gene expression of the CMV promoter is strong in vitro, but the efficiency of in vivo expression in the liver is weak. For the specific expression of a target gene in liver hepatocytes, TBG, transthyretin, and α1 antitrypsin promoters with ApoE enhancers are used. A plasmid with a liver-specific TBG promoter is available from Addgene (#61593). Although the CAG promoter is also capable of strong gene expression in the liver, the entire length of the CAG promoter (1.7 kb) cannot be incorporated into one AAV vector with the SaCas9.

2. After purification, a plasmid containing ITR sequences should be checked with SmaI or MscI to confirm that the plasmid contains intact ITRs.
3. AAV vector production by a helper-free method requires three plasmids, including a plasmid harboring a gene of interest between ITRs, the helper plasmid containing some adenovirus sequences, and a plasmid expressing the Rep and capsid gene. The capsid sequence determines the serotype of the AAV vector. The AAVpro® Helper-Free System (Takara Bio: 6630, 6650, 6651, 6673) includes the pAAV-CMV Vector, pHelper (Helper plasmid) Vector, and pRC (Rep/Cap) Vector to produce AAVs using the helper-free method. The kit includes pHelper (helper plasmid) and pRC (Rep/Cap). AAV8, AAV9, and rh10 are excellent for gene transfer to the mouse liver. Rep/Cap expression plasmids for each are available from Addgene (pAAV2/8, #112864; pAAV2/9n, #112865; pAAV2/rh10, #112866).
4. Before the dish coating, the solutions are diluted tenfold with 1 mM HCl and filtered through a 0.22 μm filter.
5. Culture dishes and plates are coated with type I collagen.
6. The DNA is also simply isolated for PCR analysis by Simple-Prep™ reagent for DNA (Takara Bio).
7. T7 endonuclease can recognize mismatch sequence (base loss, insertion, or mutation) at the DSB site induced by Cas9.
8. Design primers so that the PCR product flanks the gRNA site. When confirmed by direct visualization in gel electrophoresis, 600 bp or more PCR products are easily observed as truncated products. For microchip electrophoresis, 300–400 bp is preferable, considering the subsequent analysis by next-generation sequencers.
9. As the number of cell passages increases, the efficiency of virus production decreases. Therefore, we usually change the cells before 30 passages or decrease the AAV production.
10. We stored 10 mL dispense stocks at −30 °C. Once defrosted for the transduction, the solution is stored at 4 °C.
11. Ultracentrifugation using cesium chloride or OptiPrep® is used to purify a large quantity of highly purified AAV vectors [10]. Additionally, AAV vectors required for mouse experiments can be purified using the kit provided by Takara Bio without any specified technique. Although the AAV vector purified by the kit contains a certain number of empty vectors [11], it may not influence the functional viral titer for the experiment.
12. Reference standards for the titration are provided from ATCC (Recombinant Adeno-associated virus 2, VR-1616;

Recombinant Adeno-associated virus 8, VR-1816). We recommend employing the reference standard for assay development.

13. Since we usually use AAV vectors harboring SV40 polyA sequence (SV40pA), we target the SV40pA sequence for qPCR. You should set a specific primer set and probe for the target AAV. AAV titer assay kits targeting ITR sequence such as the AAVpro® Titration Kit (for Real Time PCR) Ver. 2 (Takara Bio) are also available as a reasonable way to quantify the titer of AAV vectors. If you want your titration method to save cost, you can refer to the method provided by Addgene (https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/). The assay employs qPCR to measure the ITR sequence by SYBR Green.
14. When administered to neonates, the AAV vector is diluted as the liver cells divide, and the efficiency of gene expression by the AAV vector is attenuated. However, the effect of gene editing by Cas9 will be sustained. The age of the mice should be selected according to their purpose. You can reduce the vector dose in the experiment on the young mouse.
15. Use hook-free tweezers. Separate the scissors used to cut the thread from the scissors used to cut the skin to keep the scissors sharp.
16. A blood sample for analysis should be selected according to the purpose of the experiment. For example, in our laboratory, we use a 3.8% sodium citrate solution (pH 7.0–8.5) as an anticoagulant and collect plasma to measure coagulation factors. However, no anticoagulant is needed in the case of serum collection.
17. SaCas9 can also adequately cut targets with PAMs of the form NNGRR(N). See the user manual from Addgene for details. (https://media.addgene.org/data/plasmids/61/61591/61591-attachment_it03kn5x5O6E.pdf).
18. For more information on the cloning of guide sequences, see the following Addgene protocol (https://media.addgene.org/data/plasmids/62/62988/62988-attachment_i-jdFt6Gm-ft.pdf).
19. The TLR3 cell line does not grow at 37 °C.
20. For details on transfection, see the Lipofectamine 3000 protocol (https://tools.thermofisher.com/content/sfs/manuals/lipofectamine3000_protocol.pdf).
21. Three plasmids are required for AAV production: (1) pHelper, (2) the plasmid harboring Rep/Cap, and (3) the plasmid containing a transgene cassette between ITR sequences. Each

plasmid used for transfection requires 23 μg/T225-flask (*see* Fig. 2).

22. The cell pellets can be stored at −30 °C until the vector purification.
23. Pluronic™ F68 Non-ionic Surfactant (final concentration of 0.001%) is added to avoid the adhesion of the AAV vector to the tube wall.
24. We usually obtain 3–7 × 10^{11} vg AAV vector from one T225 flask. If you require a high amount of the vector, ultracentrifugation using cesium chloride or OptiPrep® can be used to purify the AAV vector [10].
25. A serial dilution of the linearized plasmid containing the sequence for the primers and probe is used to determine the standard curve for the quantification.
26. Calculate the copy number of the linearized plasmid from the concentration and molecular weight of the plasmid. The concentration of the plasmid is measured by an absorbance spectrophotometer, and the formula weight of the plasmid is calculated as the molecular weight of 1 bp of DNA as 660. The copy number of the linearized plasmids can be determined using the following formula: (copy/μL) = (concentration (ng/μL)/F.W.) × 6.02 × 10^{14}.
27. The threshold should be placed in the region of exponential phase. To standardize threshold placement, we recommend you adjust the threshold until the 10^8 copies standard reads ~13.08 Cts. The replicates should be tight, within 0.5 Cts (threshold cycle). The standard curve should be linear over the entire range of where you expect your unknowns to fall. The standard curve should have a correlation coefficient of at least 0.99 and an amplification efficiency of 95–105%. Cts of negative controls (H_2O) should be above 35. According to the dilution of the sample and volume of sample loaded, calculate the number of copies per mL (vg/mL). Since real-time PCR only targets one of the two strands packaged within the AAV capsid, a multiplication factor of 2 is also required. For detailed information on AAV titer analysis, see the following protocol (https://www.isbiotech.org/ReferenceMaterials/pdfs/AAV8-RSM-genome-copy-titration-by-QPCR.pdf).
28. Thaw the AAV vector stored at −80 °C and dilute to the required volume with HN buffer containing Pluronic™ F68 Non-ionic Surfactant (final concentration of 0.001%). We usually use 0.3–1 × 10^{12} vector genome (vg)/mouse of AAV8 vector for efficient genome editing in the mouse liver. Vectors are placed on ice and allowed to reach room temperature before administration to mice. Vectors should be dispensed in 1.5 mL tubes per individual mouse.

29. Nothing is put into the syringe to obtain serum. Instead, add sodium citrate, EDTA, or heparin to the syringe to obtain plasma. Sodium citrate is used to measure coagulation factors in plasma.
30. Compression will easily control minor bleeding with a sterile cotton swab.

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Chapter 16

Nonviral Ex Vivo Genome Editing in Mouse Bona Fide Hematopoietic Stem Cells with CRISPR/Cas9

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and Yutaka Hanazono

Abstract

Knock-in therapy, in which an insertion site can be controlled, would be more suitable for the treatment of genetic blood disorders as compared to conventional gene therapy with lentivirus vectors that introduce genes into the genome randomly. Recent advancements in genome editing technology have substantially improved the knock-in efficiency, making it a reality. We present the details of a virus-free CRISPR/Cas9-based genome editing method for bona fide mouse hematopoietic stem cells.

Key words Genome editing, CRISPR/Cas9, Knock-in, Mouse hematopoietic stem cells, Plasmid

1 Introduction

Bona fide hematopoietic stem cells are cells that can reconstitute entire hematopoietic cells in vivo, both myeloid and lymphoid cells, for a long period (*see* **Note 1**). Inherited blood disorders, which are caused by genetic mutations in hematopoietic stem cells, can be treated by transplanting the cells from healthy donors. Especially when the mutations reduce survival or growth ability of the progenitor cells such as in X-linked severe combined immunodeficiency (X-SCID) and hemoglobinopathy, a high therapeutic effect can be obtained because transplanted, genetically corrected cells having a survival or growth advantage gradually replace the patient's endogenous sick cells. Despite the therapeutic effects, there are still considerable hurdles including the donor availability and immune responses to transplanted cells.

In gene therapy, a correct gene is delivered ex vivo into patient-derived hematopoietic stem cells using randomly genome integrative gammaretrovirus vectors, and the cells are autologously transplanted back to the patients. Although the therapy has shown its efficacy in treating genetic diseases such as X-SCID [1], it caused

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leukemia due to the integration of transgene into a proto-oncogene [2]. Although recent lentivirus-based gene therapy for X-SCID much less likely causes leukemia [3], the insertion sites cannot be controlled. In addition, the transgene is driven by an exogenous promoter, which makes the proper expression difficult.

As an alternative to the conventional gene therapy, genome editing therapy has attracted attention. In this therapy, a correct gene is "knocked in" into a specific site of the genome with genome editing nuclease such as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) (*see* **Note 2**). Genome editing therapy allows to control insertion sites and also to express a correct gene through the natural promoter. A gene is knocked in to genomic DNA via either of the two pathways, homology-directed repair (HDR) or nonhomologous end-joining (NHEJ). Although HDR is a precise (virtually an error-free) pathway, its efficiency is typically very low. On the other hand, although NHEJ is an error-prone pathway, its efficiency is much higher than that of HDR. To circumvent the issue of low accuracy in NHEJ, we inserted a correct gene into intron 1 of a mutated gene, because, if some insertions or deletions happen to be introduced into introns, such mutations in introns have little adverse effect. In addition, this method can rescue virtually all mutations with only a single plasmid knock-in vector (*see* **Note 3**). Using this method, we successfully cured X-SCID mice [4]. Here, we describe the method for genome editing in mouse hematopoietic stem cells via NHEJ that does not require any viral vectors (*see* **Note 4**). There are two major cruxes in this method: the transfection of Cas9 as Cas9/gRNA ribonucleoprotein (Cas9-RNP) (*see* **Note 5**) and the addition of homology-independent targeted integration (HITI) sequences to the plasmid knock-in vector to prevent the insertion of plasmids in the opposite direction (*see* **Note 6**).

2 Materials

2.1 Medium

1. Lysis buffer: 154.4 mM ammonium chloride, 10 mM potassium bicarbonate, and 97.3 μM ethylenediaminetetraacetic acid (EDTA).
2. PBS/BSA: 0.5% bovine serum albumin and 2 mM EDTA in PBS (−).
3. Culture medium (*see* **Note 7**): Iscove's modified Dulbecco's medium supplemented with insulin–transferrin–selenium supplement-A, 100 ng/mL murine thrombopoietin, 100 ng/mL stem cell factor, and 100 ng/mL Flt3-ligand.

2.2 Magnetic Separation

1. Magnetic cell separator such as autoMACS Pro Separator (Miltenyi Biotec).

2. Lineage cell depletion kit. For autoMACS Pro Separator, Lineage Cell Depletion Kit (Miltenyi Biotec), which contains magnetic beads binding to lineage markers of Gr-1, CD11b, B220 (CD45R), Ter119, and CD3, is recommended.

2.3 Electroporation of Genome Editing Tools

1. Electroporator. We use NEPA21 (Nepagene) because one can adjust many parameters with the apparatus to optimize electroporation conditions for each cell type.
2. Electroporation cuvette. When using NEPA21, a set of 1 mm gap NEPA Cuvettes and disposable plastic pipettes (Nepagene, EC-001S) is recommended for sorting mouse lineage-negative (Lin^-) cells.
3. Opti-MEM.
4. Cas9 protein.
5. gRNA: crRNA and tracrRNA.
6. Duplex buffer: 30 mM HEPES and 100 mM potassium acetate (pH 7.5).
7. Knock-in construct: Circular plasmid vector with cDNA.
8. Softwares for designing crRNA such as Benchling (https://benchling.com/), CCTop (http://crispr.cos.uni-heidelberg.de) [5], and CRISPRdirect (http://crispr.dbcls.jp) [6].
9. Softwares for designing PCR primer sets. We usually use Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) because it can design primers with relatively high specificity by BLAST search.

2.4 Evaluation of Genome Editing Efficiency

1. Colony-forming unit (CFU) assay kit. The gold standard is MethoCult GF M3434 (STEMCELL Technologies).
2. Software for analyzing double-strand break (DSB) efficiencies: TIDE (http://shinyapps.datacurators.nl/tide/) [7].

2.5 Transplantation

1. Irradiation equipment.
2. One milliliter syringe (29G).

2.6 Peripheral Blood Collection

1. EDTA blood collection tube.

3 Methods

3.1 Preparation of Mouse Lin^- Cells

1. Dissect out the tibia and femurs from six- to ten-week-old male mice and place them in a 60 mm dish containing PBS/BSA. Cut off both ends of each bone and flush out the bone marrow

with PBS/BSA using a 23G needle on a 1 mL syringe. Disaggregate the bone marrow tissues by repeated aspiration using the needle.

2. Transfer the cell suspension to a 50 mL centrifuge tube and add 20 mL of PBS/BSA. Centrifuge the suspension at 820 × *g* for 5 min at 4 °C. Remove the supernatant, add 2–3 mL of lysis buffer, and incubate it for 5 min at room temperature. Centrifuge it again at 820 × *g* for 5 min at 4 °C. Resuspend the cells with 20 mL of PBS/BSA and filter the suspension with a 70 μm cell strainer.
3. Label the cells with magnetic beads for lineage markers according to the manufacturer's instruction. For instance, when using Lineage Cell Depletion Kit, adjust the cell concentration to 1×10^7 cells/mL with PBS/BSA. Add magnetic beads for all lineage markers (10 μL each) to 1×10^7 cells, and incubate it for 20 min at 4 °C. Wash the cells with PBS/BSA and centrifuge them at 820 × *g* for 10 min at 4 °C. Resuspend them with 500 μL of PBS/BSA at a concentration of 2×10^7 cells/mL.
4. Isolate Lin^- cells with a magnetic cell separator. For instance, when using autoMACS Pro Separator, apply the "Deplete" program according to the manufacturer's instruction.
5. Culture Lin^- cells with the culture medium for 24 h at the concentration of 1×10^6 cells/mL in 24-well plate.

3.2 Preparation of Genome Editing Tools

1. Designing of specific crRNA for intron 1 of a target gene. Using several web-based softwares such as Benchling, CCTop, and CRISPRdirect, design crRNA with high efficiency and specificity scores. We recommend designing three gRNAs for each target site.
2. Preparation of Cas9-RNP. Resolve crRNA and tracrRNA at 200 μM with duplex buffer. Mix the two RNA oligos in equimolar concentrations to a final duplex concentration of 44 μM. To form the crRNA:tracrRNA duplex, heat the mixture at 95 °C for 5 min, and remove it from heat and allow it to cool to room temperature on a benchtop. Then, mix the duplex (final 60 μM) with Cas9 protein (final 60 μM) in Opti-MEM and incubate it for 20 min at room temperature.
3. Construction of knock-in vector (Fig. 1). Clone codon-optimized correct cDNA without exon 1 to cloning plasmid such as pUC19 and confirm the sequence. Codon optimization does not only make the translation more efficient but also make the detection of knock-in cells by PCR easier. Add HITI sequences that are complemental to crRNA plus PAM at both ends of cDNA [8]. Prepare the plasmid at 1 μg/μL. The vector can rescue any mutations except those present in exon 1.

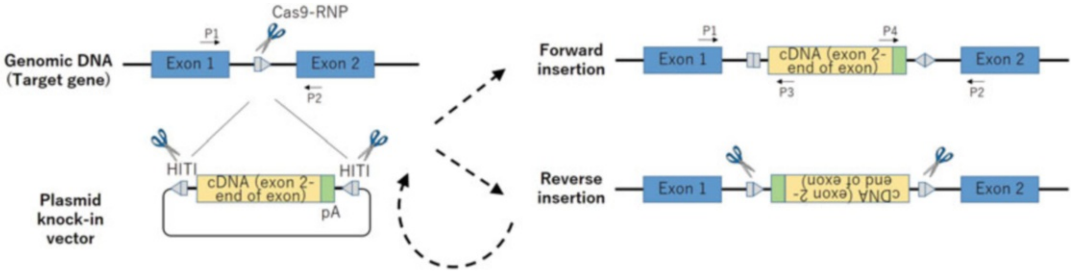

Fig. 1 A schematic illustration of cDNA knock-in using the plasmid vector. cDNA lacking exon 1 is knocked in to intron 1 of a target gene. On both ends of cDNA, complementary strands of crRNA + PAM (called HITI) are added. The HITI sequences enhance knock-in efficiencies via NHEJ by twice because the knock-in in the reversed orientation is cut again by Cas9-RNP. Primer sets for genotyping are also shown (for DSB detection, P1 + P2, and for knock-in detection, P1 + P3 and P4 + P2)

4. Designing primers for genotyping (Fig. 1) using Primer-BLAST. For knockout cells, design primer sets outside the CRISPR/Cas9 target site. For knock-in cells, design primer sets for 5′ insertion (forward, outside of target site, and reverse, inside of codon-optimized cDNA) and 3′ insertion (forward, inside of codon-optimized cDNA, and reverse, outside of target site).

3.3 Genome Editing of Mouse Lin$^-$ Cells Using NEPA21

1. Harvest cultured mouse Lin$^-$ cells and count a cell number. Centrifuge the cells at 100 × *g* for 10 min at 4 °C. Remove the supernatant and resuspend it with Opti-MEM. Wash the cells again with Opti-MEM.
2. Mix 2 × 10^5 cells with Cas9-RNP (final 1 μM) and knock-in vector (2 μg) in 20 μL. When you also want to evaluate DSB-alone efficiencies for the purpose of choosing the best crRNA, mix the cells only with Cas9-RNP.
3. Transfer all of 20 μL to a 1 mm gap electroporation cuvette.
4. Set parameters of NEPA21 as follows (*see* **Note 8**): for poring pulse, 100 V, pulse length 5 ms, pulse interval 50 ms, pulse number eight times, decay rate 10%, and polarity +. For transfer pulse, 20 V, pulse length 50 ms, pulse interval 50 ms, pulse number five times, decay rate 40%, and polarity +/−. Place the cuvette in the electroporation chamber, and check the resistance of cuvettes. Appropriate is 70–90 Ω.
5. Apply the pulse to the electroporation cuvette.
6. Add 100 μL of the culture medium into the cuvette.
7. Aspirate the cell suspension gently using a disposable plastic pipette supplied along with the cuvette.
8. Proceed to either of Subheadings 3.4, 3.5, or 3.6.

3.4 Evaluation of DSB Efficiency In Vitro

1. Culture the electroporated cells in a 3.5 cm dish using a CFU assay kit such as MethoCult GF M3434 for 7–10 days.
2. Calculate CFUs based on the numbers of colonies. Also evaluate the differentiation ability of transfected cells by counting colonies derived from erythroid progenitor cells (BFU-E), granulocyte-macrophage progenitor cells (CFU-GM, CFU-G, and CFU-M), and multipotential granulocyte, erythroid, macrophage, and megakaryocyte progenitor cells (CFU-GEMM) according to the manufacturer's instruction of a CFU assay kit.
3. Analyze DSB efficiencies using TIDE. Briefly, harvest the cells and extract DNA. Perform PCR and Sanger sequencing for both transfected and untransfected cells. Upload a sequencing file with the extension "ab1" or "scf" to the TIDE website.

3.5 Evaluation of Knock-in Efficiency In Vitro

1. Culture the electroporated cells in a 3.5 cm dish using a CFU assay kit such as MethoCult GF M3434 for 7–14 days.
2. Calculate CFUs and evaluate the differentiation ability of transfected cells as described in Subheading 3.4, **step 2**.
3. Analyze the knock-in efficiencies. Pluck well-separated individual colonies with a P10 pipette and transfer each colony into a PCR tube. Identify colonies consisting of knock-in cells by PCR, and you can tell the knock-in efficiency which is the ratio of the number of knock-in colonies divided by the total number of colonies.

3.6 Evaluation of Genome Editing In Vivo

1. Suspend electroporated cells with the culture medium.
2. Load the cell suspension (1×10^6 cells/200 μL) into a 1 mL syringe fitted with a 25G needle.
3. Irradiate NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/ShiJic (NOG) mice (*see* **Note 9**) with 2.5 Gy (*see* **Note 10**).
4. Under general anesthesia of the irradiated mice, incise the neck and inject the cell suspension into the jugular vein. Suture the incision.
5. Every 2 weeks after transplantation, aspirate 100 μL of peripheral blood from the jugular or tail vein under general anesthesia, and transfer it into EDTA blood collection tubes.
6. Evaluation of genome editing efficiency of the peripheral blood cells that should reflect that of hematopoietic stem cells. PCR is a common method to examine the efficiency. If genome-edited cells can be detected by flow cytometry, it is easier. Careful attention should be paid when evaluating the efficiency because some target genes have a survival or growth advantage. For example, when the correct cDNA (exon 2–8) of *Il2rg* is knocked in to Lin$^-$ cells of *Il2rg* knockout mice that originally

lack lymphocytes (Fig. 1), only genome-edited lymphocytes will appear in the peripheral blood after transplantation, resulting in a strongly positive bias on the efficiency data when you examine the genome editing efficiency on lymphocytes. In this case, the bias can be avoided by measuring the efficiency in myeloid cells, which are not affected by the growth advantage.

4 Notes

1. Bona fide hematopoietic stem cells, also called long-term hematopoietic stem cells, are cells that can repopulate long-term in vivo after the transplantation. There are also short-term hematopoietic stem cells that can repopulate only short term. Therefore, the evaluation of genome editing efficiencies in long-term (bona fide) hematopoietic stem cells, and serial transplantation, secondary or even tertiary one, is necessary to exclude the bias (usually positive one) brought by short-term hematopoietic stem cells included in the transplanted cells. In addition, so-called hematopoietic stem cell fractions such as Lin^- cells mentioned in this section also include hematopoietic "progenitor" cells that are differentiated from hematopoietic "stem" cells. Short-term hematopoietic stem cells and hematopoietic progenitor cells are more cycling, and they are easier to be genome-edited than hematopoietic stem cells. Therefore, it should be noted that the genome editing efficiency of hematopoietic stem cell fractions (such as Lin^- cells) containing short-term hematopoietic stem cells and hematopoietic progenitor cells are usually much higher than that of bona fide hematopoietic stem cells themselves.
2. An interim report on experimental human genome editing therapy for sickle cell disease and β-thalassemia was recently published [9]. In the paper, BCL11A, which produces the fetal-type globin and suppresses the adult-type globin, was knocked out. As a result, the normal fetal-type globin gradually replaced the sick adult-type globin, greatly ameliorating the patients' signs and symptoms. However, there are few diseases that can be successfully treated in the same way (i.e., gene knockout) as reported. The development of efficient knock-in technology is crucial for the treatment of most genetic diseases.
3. This therapy can rescue any mutations except those present in exon 1 because cDNA without exon 1 is knocked in to intron 1 of a target gene via NHEJ (Fig. 1).
4. For knock-in via homology-directed repair, not NHEJ, adeno-associated virus 6 would be better than plasmid as donor vectors [10].

5. DSB efficiency in mouse Lin$^-$ cells with Cas9-RNP (26% in our case) was much higher than the efficiency with Cas9-expressing plasmid (9%). The efficiency in bona fide hematopoietic stem cells using Cas9-RNP was 15–45% assessed by tertiary transplantation experiments (*see* **Note 1**), although it was still lower than that in cell lines such as mouse embryonic stem cells (at higher than 98%).
6. HITI is a complemental sequence of crRNA plus PAM. Addition of HITI sequences at both ends of cDNA theoretically doubles the knock-in efficiency via NHEJ, because cDNA is recut by Cas9 when cDNA is inserted in the opposite direction (Fig. 1) [4, 8].
7. Culture medium is important. There is controversy as to whether the conventional medium expands bona fide hematopoietic stem cells. It may rather spoil their abilities of engraftment and multipotency. Recently, mouse bona fide hematopoietic stem cells expanded hundreds of times over a month in a medium containing polyvinyl alcohol (PVA) and a low concentration of stem cell factor (SCF) as estimated by limiting dilution analysis [11]. When you want to propagate bona fide hematopoietic stem cells for longer than 4 days, it should be better to use the PVA-/SCF-containing medium. In the method described here, however, the ex vivo culture period is not that long and the medium described here works well enough.
8. Although there are various types of electroporators, a trade-off between transfection efficiency and cell viability is observed. Therefore, it is important to find the optimal conditions that can achieve high transfection efficiency while maintaining a high survival rate as possible. When optimizing parameters of NEPA21, the voltage, pulse length, and pulse number for poring pulse are critical to achieve the high transfection efficiency and survival rate. The conditions described here are kind of optimal in our laboratory.
9. Usually, CD45 isoforms (NOG, CD45.1, C57BL/6, CD45.2) are used to distinguish the donor and host cells.
10. The dose of radiation depends on the strain of recipient mice. For example, C57BL/6 mice are usually irradiated with 9.5 Gy. On the other hand, NOG mice are irradiated with 2.5 Gy because they are radiosensitive due to the knockout of *Prkdc* that is one of the key factors of DSB repair. The irradiation condition described here is an intensity-reduced one, instead of a myeloablative one, considering survival rates of irradiated mice.

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Chapter 17

Genome Editing of Rat

Takehito Kaneko

Abstract

Many genetically engineered rat strains have been produced by the development of genome editing technology, although it used to be technical difficulty and low production efficiency. Knockout and knock-in strains can be simple and quick produced using zinc finger nuclease (ZFN), *transcription activator-like effector nuclease* (*TALEN*), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. Presently, genome edited strains have been produced by microinjection and a new electroporation method named technique for animal knockout system by *electroporation* (*TAKE*). This chapter presents the latest protocols for producing genome edited rats.

Key words Rat, CRISPR-Cas9, In vitro fertilization, Embryos, Microinjection, Electroporation, Embryo transfer

1 Introduction

Many genetically engineered rat strains have been already supplied worldwide using genome editing technology. Simple and quick production of knockout and knock-in rats is possible using zinc finger nuclease (ZFN), *transcription activator-like effector nuclease* (*TALEN*), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 [1–4] without embryonic stem (ES) cells or induced pluripotent stem (iPS) cells [5]. Microinjection method, which directly introduces endonucleases into pronuclear stage embryos, has been used in the production of new genome edited strains [6]. Although this is a gold standard tool, it requires a high skill level to minimize cell damage during injection. We developed new nuclease introduction system into embryos by electroporation to satisfy quick supply of genome edited rats. Technique for animal knockout system by electroporation (TAKE) can be applied to introduce nucleases into intact embryos without weakening zona pellucida by using a three-step electrical pulse system [7, 8]. TAKE method is possible to produce genome edited animals without

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specialist skills, such as conventional microinjection method. This chapter presents the latest protocols of microinjection and TAKE method for the production of genome edited rats.

2 Materials

2.1 Preparation of Pronuclear Stage Embryos by Natural Mating

1. Mature male and female rats.
2. Modified Krebs-Ringer bicarbonate (mKRB) medium for rat embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C (*see* **Note 1**).
3. Sterile mineral oil.
4. 30–60 mm plastic culture dish.
5. CO_2 incubator.
6. Pregnant mare serum gonadotropin (PMSG).
7. Human chorionic gonadotropin (hCG).
8. Syringe (1 mL) with 30G steel needle.
9. Pair of small scissors.
10. Fine-tipped forceps.
11. Glass capillary pipettes.

Table 1
Components of mKRB medium

Components	mKRB mg/100 mL (mM)	HTF mg/100 mL (mM)
NaCl	553 (94.6)	594 (101.6)
KCl	36 (4.8)	35 (4.7)
$CaCl_2$	19 (1.7)	23 (2.0)
$MgSO_4 \cdot 7H_2O$	29.3 (1.2)	5 (0.2)
KH_2PO_4	16 (1.2)	5 (0.4)
$NaHCO_3$	211 (25.1)	210 (25.0)
Na-lactate (60% syrup)	0.19 mL	0.34 mL
Na-pyruvate	6 (0.5)	4 (0.3)
D-glucose	100 (5.6)	50 (2.8)
Penicillin G	7	7
Streptomycin	5	5
Bovine serum albumin (BSA)	400	400

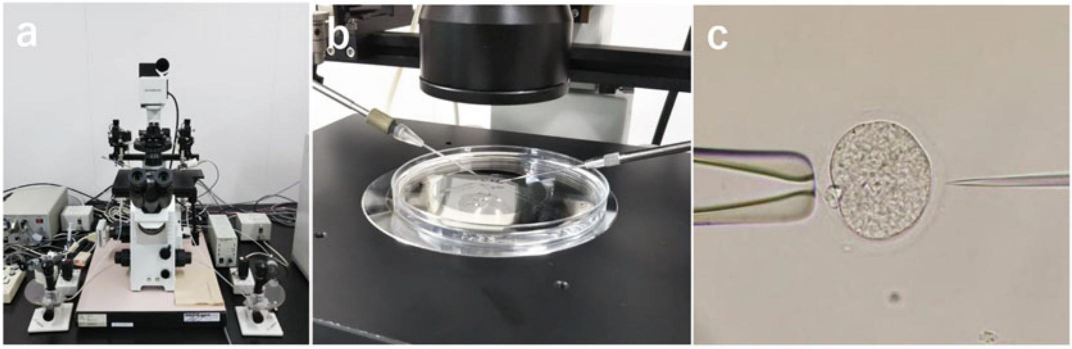

Fig. 1 (**a**) Micromanipulator. (**b**) Setup for microinjection. (**c**) Injection of nucleases into pronuclear of embryo. (The part of figure is reproduced from Ref. [6])

2.2 Preparation of Pronuclear Stage Embryos by In Vitro Fertilization (IVF)

1. Mature male and mature or immature female rats.
2. Human tubal fluid (HTF) for mouse and rat in vitro fertilization (IVF) and embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C (*see* **Note 1**).
3. Sterile mineral oil.
4. 30–60 mm plastic culture dish.
5. CO_2 incubator.
6. Pregnant mare serum gonadotropin (PMSG).
7. Human chorionic gonadotropin (hCG).
8. Syringe (1 mL) with 30G steel needle.
9. Pair of small scissors.
10. Fine-tipped forceps.
11. Glass capillary pipettes.

2.3 Microinjection of Nucleases into Pronuclear Stage Embryos (Microinjection Method)

1. Micromanipulator (Fig. 1a).
2. Micropipette puller (Sutter Instrument, Novato, CA, USA).
3. Microforge.
4. Glass capillary pipettes.
5. Cas9 protein, tracrRNA, crRNA designed target gene, and ssODN (Integrated DNA Technologies, Inc. Coralville, IA, USA).
6. Distilled water.
7. Loading tips.
8. mKRB medium.
9. Sterile mineral oil.

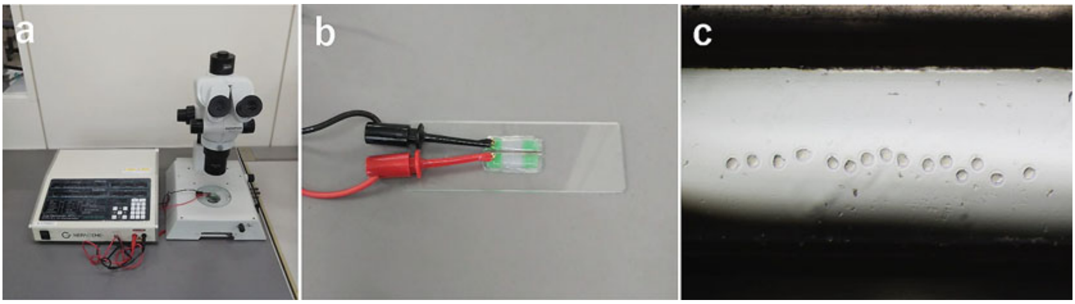

Fig. 2 (**a**) Electroporator NEPA 21. (**b**) 1 mm gap glass slide electrodes (CUY501P1-1.5). (**c**) Embryos are introduced between the electrodes filled with nuclease solution. (The part of figure is reproduced from Ref. [9])

10. 10 cm plastic culture dish.
11. CO_2 incubator.

2.4 Electroporation of mRNAs into Intact Pronuclear Stage Embryos (TAKE Method)

1. Electroporator (NEPA 21: NEPA GENE Co. Ltd., Chiba, Japan) (Fig. 2a) (*see* **Note 2**).
2. Glass slide electrodes (1 mm gap; CUY501P1-1.5: NEPA GENE Co. Ltd.) (Fig. 2b) (*see* **Note 3**).
3. Cas9 protein.
4. tracrRNA for the target.
5. Single-strand oligo donor (ssODN).
6. Opti-MEM medium (Thermo Fisher Scientific).
7. Glass capillary pipettes.

2.5 Embryo Transfer

1. Mature female rats.
2. Vasectomized male rats.
3. Isoflurane for anesthesia (2%, 1 L/min).
4. Pair of small scissors.
5. Fine-tipped forceps.
6. Glass capillary pipettes.
7. 30G steel needle.
8. Wound clips.

2.6 Genotyping of Delivered Pups

1. Pair of small scissors.
2. FTA cards.
3. DNA extraction kit.
4. PCR system.
5. Electrophoresis system.
6. DNA sequencing system.

3 Methods

3.1 Preparation of Pronuclear Stage Embryos by Natural Mating

1. Use mKRB medium to manipulate embryos.
2. Induce superovulation in females by intraperitoneal injection of 150–300 IU/Kg PMSG, followed by injection of 75–300 IU/Kg hCG 48 h later.
3. After hCG injection, mate females with males overnight.
4. Confirm the presence of vaginal plugs to ensure mating has occurred.
5. Prepare four 50 μL drops of mKRB medium in the culture dish.
6. Cover drops with mineral oil in the culture dish.
7. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.
8. Euthanize females by CO_2 overdose and cervical dislocation.
9. Remove the oviducts using a pair of small scissors (*see* **Note 4**).
10. Flush ampulla with mKRB medium using a 1 mL syringe with a 30G steel needle.
11. Collect pronuclear stage embryos using a glass capillary pipette and transfer to one of the four drops of mKRB medium.
12. Remove cumulus cells and other debris, and transfer the embryos to another drop of mKRB medium (*see* **Note 5**).
13. Place the pronuclear stage embryos in the fourth drops of mKRB medium, and place at 37 °C under 5% CO_2 and 95% air until used for microinjection or electroporation.

3.2 Preparation of Pronuclear Stage Embryos by In Vitro Fertilization (IVF)

1. Prepare two culture dishes with 200 μL drops of HTF medium covered with sterile mineral oil.
2. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.
3. Euthanize a male by CO_2 overdose and cervical dislocation.
4. Remove the cauda epididymides using a pair of small scissors.
5. Squeeze the sperm mass out of the epididymides using fine-tipped forceps.
6. Place the sperm mass in drops of HTF medium (*see* **Note 6**).
7. Capacitate sperm for 60 min at 37 °C under 5% CO_2 and 95% air.
8. Induce superovulation in females by intraperitoneal injection of 150–300 IU/Kg PMSG, followed by injection of 75–300 IU/Kg hCG 48 h later.
9. Euthanize the females by CO_2 overdose and cervical dislocation, 13–15 h after hCG injection.

10. Remove the oviducts using a small pair of scissors (*see* **Note 4**).
11. Place the oviducts into mineral oil in another culture dish.
12. Collect the cumulus-oocyte complexes by puncturing the ampulla of oviducts using a steel needle.
13. Transfer the cumulus-oocyte complexes to drops of HTF medium.
14. Add the capacitated sperm suspension into the drops of HTF medium with the oocytes. The final sperm concentration is approximately 5×10^5 cells/mL.
15. Place the culture dish at 37 °C under 5% CO_2 and 95% air.
16. Collect the embryos that appear pronuclear 5 h after insemination using glass capillary pipettes.
17. Place the pronuclear stage embryos in a new drop of HTF medium at 37 °C under 5% CO_2 and 95% air until used for microinjection or electroporation.

3.3 Microinjection of Nucleases into Pronuclear Stage Embryos (Microinjection Method)

1. Prepare 10 μL nuclease solution at 20 μg/mL for Cas9 protein and 50 μg/mL dualRNA (equal molar mixture of crRNA and tracrRNA) in distilled water (*see* **Note 7**).
2. Add 10–50 μg/mL ssODN in the nuclease solution when producing knock-in strain.
3. Prepare injection and holding pipettes using a micropipette puller and a microforge.
4. Introduce 2–3 μL of nuclease solution into the tip of the injection pipette using the loading tip.
5. Mount the injection pipette in the right pipette holder of the micromanipulator (Fig. 1b).
6. Mount the holding pipette in the left pipette holder of the micromanipulator (Fig. 1b).
7. Prepare small 10–20 μL drops of mKRB medium covered with sterile mineral oil in the 10 cm plastic culture dish (Fig. 1b).
8. Transfer embryos to the drops of mKRB medium.
9. Transfer an embryo to the holding pipette and stabilize (Fig. 1b).
10. Inject 2–3 pl of mRNA solution into the male pronucleus or cytoplasm (Fig. 1c).
11. Culture embryos after injection in 100 μL drops of mKRB medium covered with sterile mineral oil at 37 °C under 5% CO_2 and 95% air until embryo transfer.

Table 2
Recommended pulse setting using 1 mm gap glass slide electrodes (CUY501P1-1.5)

	Voltage (V)	Pulse width (ms)	Pulse interval (ms)	Number of pulses	Decay rate (%)	Polarity
Poring pulse	40	3.5	50	4	10	+
Transfer pulse	15	50	50	5	40	+/−

Table 3
Development of rat embryos introduced into nucleases by microinjection or TAKE method

Methods	No. of embryos examined	No. of two-cell embryos (%)	No. of offspring (%)	No. of knockout offspring (%)
Microinjection	40	19 (48)	13 (68)	10 (77)
TAKE	25	25 (100)	17 (68)	17 (100)

The data are reproduced from Ref. [9]

3.4 Electroporation of mRNAs into Intact Pronuclear Stage Embryos (TAKE Method)

1. Prepare 10 μL nuclease solution at 100 μg/mL for Cas9 protein and 500 μg/mL dualRNA (equal molar mixture of crRNA and tracrRNA) in Opti-MEM medium (*see* **Note 7**).
2. Add 100 μg/mL ssODN in the nuclease solution when producing knock-in strain.
3. Introduce 10 μL nuclease solution into the electrode (Fig. 2b).
4. Place 10–20 pronuclear stage embryos in a line between electrodes (Fig. 2c).
5. Connect the electrodes to the electroporator (Fig. 2a).
6. Set up the poring and transfer pulses (Table 2) on the electroporator.
7. Electroporate the intact embryos (*see* **Notes 8–10**).
8. Place electroporated embryos into fresh drops of mKRB medium at 37 °C under 5% CO_2 and 95% air until embryo transfer (Table 3).

3.5 EmbryoTransfer

1. Mate females with vasectomized males on the day before transfer.
2. Confirm the presence of vaginal plugs to ensure mating has occurred (*see* **Note 11**).
3. Anesthetize a female using isoflurane.
4. Expose the ovary, oviduct, and part of the uterus through an abdominal incision.

5. Make a small hole in the upper ampulla using a sharp 30G needle.
6. Aspirate 5–10 two-cell embryos into a glass capillary pipette with a few small air bubbles (*see* **Note 12**).
7. Insert this capillary pipette into the hole in the oviduct.
8. Transfer the embryos with air bubbles.
9. Return the ovary, oviduct, and uterus back inside the body cavity and seal the incision with wound clips.
10. Transfer more embryos with the same procedure into another oviduct.
11. Deliver pups at 21 days of gestation.

3.6 Genotyping of Delivered Pups

1. Collect blood from three-week-old pups.
2. Blot blood samples onto FTA cards.
3. Extract genomic DNA from blood samples using a DNA extraction kit.
4. Amplify the target sequences by PCR.
5. Electrophorese the PCR products on agarose gels.
6. Sequence the PCR products to confirm the mutations (*see* **Note 13**).

4 Notes

1. All media can be stored for up to 1 month.
2. The device of other companies can be used, although electrical conditions were different.
3. 5 mm gap glass slide electrodes (CUY520P5: NEPA GENE Co. Ltd.) are easy to manipulate embryos.
4. Collect oviducts immediately after euthanasia to avoid physiological damage to oocytes.
5. If cumulus cells are attached to embryos, remove using 0.1% hyaluronidase.
6. Handle gently to maintain high sperm motility.
7. Should be made up nuclease solution for each experiment.
8. Embryo derived from freeze-dried and frozen sperm and frozen embryo can be used for TAKE method [10–13].
9. TAKE method can use intact embryos without any chemical treatment, such as zona thinning using Tyrode's acid solution.
10. Nucleases introduced into embryos by TAKE method can be confirmed using fluorescence nucleases [14].

11. Pseudopregnancy can be introduced using sonic vibration instead of vasectomized male [15].
12. Embryo transfer at the two-cell stage is suitable for subsequent embryo development.
13. Typical results for the production of knockout rats by the microinjection or electroporation of endonucleases are shown in Table 2.

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Chapter 18

A Simple and Efficient Method for Generating KO Rats Using In Vitro Fertilized Oocytes

Kohtaro Morita, Arata Honda, and Masahide Asano

Abstract

The development of ZFN, TALEN, and CRISPR/Cas9 systems has simplified the process of generating knockout (KO) and knock-in (KI) rats in addition to mice. However, in rats, an efficient genome editing technique that uses in vitro fertilized oocytes has not been established. Recently, we reported the stable generation of offspring from five standard strains of rats by superovulation and in vitro fertilization (IVF). Furthermore, genome-edited rats can be easily generated by electroporation. First, juvenile female rats are administered LHRH (luteinizing hormone-releasing hormone) to synchronize the estrous cycle and then AIS (Automatic Identification System) with PMSG (pregnant mare serum gonadotropin) before hCG (human chorionic gonadotropin) for superovulation. Sperm collected from a sexually mature male rat the following morning is then pre-cultured. Cumulus cell-oocyte complexes (COCs) are collected from female rats under anesthesia, and COCs are induced into a medium containing concentration-adjusted sperm. Thereafter, oocytes with two pronucleus are selected as fertilized oocytes. Next, fertilized oocytes are transferred into a glass chamber containing CRISPR ribonucleoprotein (RNP) complexes formed from gRNA and Cas9 protein. After electroporation, fertilized oocytes are then immediately transferred to culture medium. The next day, embryos are transferred into the oviduct of pseudopregnant female rats. Using the above method, offspring can be obtained 22 days after the day of embryo transfer. In this paper, we outline a method allowing simple and efficient generation of genetically modified rats without the need for technically difficult micromanipulation techniques.

Key words CRISPR/Cas9, Electroporation, Genome editing, In vitro fertilization, Rat

1 Introduction

The generation of genetically modified animals is an important component of life science and basic clinical research. Because mouse is the first experimental animal in which ES (embryonic stem) cells were generated, many strains of KO/KI mice have been produced. The development of the CRISPR/Cas9 system in 2012 has made the generation of KO/KI more feasible in organisms other than mice [1]. Rat represents one of the most versatile experimental animals in the fields of neuroscience, pharmacology,

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nutrition science, hypertension, diabetes, and organ transplantation research. Therefore, genome editing technology is expected to have significant utility in rats. Furthermore, electroporation technology has made it possible to induce gRNA and Cas9 mRNA into fertilized oocytes, thereby easily producing genome-edited rats without the use of micromanipulation techniques [2]. Subsequently, numerous strains of genome-edited rats generated using the CRISPR/Cas9 system have continued to be deposited in the National BioResource Project-Rat (NBRP-Rat) in Japan (http://www.anim.med.kyoto-u.ac.jp/nbr/Default.aspx) and Rat Resource & Research Center (RRRC) in the USA (https://www.rrrc.us/), with these rats then being supplied to many researchers. Although previous studies have reported the successful generation of rat offspring from in vitro fertilized oocytes [3–7], genome-edited rats have been derived from fertilized oocytes by natural mating in many other studies [2, 8–13], which reflects the technical difficulty of efficiently producing rats by IVF. In addition, improved genome editing via oviductal nucleic acid delivery (*i*-GONAD) for rats has been established as a method that does not require IVF and embryo transfer [13]. However, it is difficult to KI a long DNA fragment using this method. Furthermore, the use of strains with poor reproductive efficiency further limits the generation of genome-edited rats. Therefore, there is a need for methods allowing the stable generation of genome-edited rats derived from a large number of in vitro fertilized oocytes. Recently, we reported that offspring can be stably obtained from five standard rat strains by IVF [14]. Surprisingly, indel mutation was induced at the target site of all rats by transferring RNP complexes into in vitro fertilized oocytes using an electroporator [14].

This paper describes a simple method of producing genome-edited rats using in vitro fertilized oocytes without microinjection techniques on the basis of our previous report [14]. We believe that our method represents a simple and effective technique for generating genetically modified rats requiring only the manipulation of embryos with a mouth pipette.

2 Materials

2.1 Animals

1. Juvenile female rats for super ovulation (3.5–4.5 weeks old, 3–5 rats).
2. A sexually mature male rat for the collection of spermatozoa (over 10 weeks old).
3. Sexually mature vasectomized rats (Wistar strain rats recommended) for preparing recipient rats (over 10 weeks old, 4–6 rats) (*see* **Note 1**).

4. Sexually mature female rats (Wistar strain rats recommended) as recipient rats for embryo transfer (over 8 weeks old, 4–6 rats).
5. Sexually mature female rats (Wistar strain rats recommended) for preparing foster rats (over 8 weeks old, 2 rats).
6. Sexually mature male rats (Wistar strain rats recommended) for preparing foster rats (over 10 weeks old, 2 rats).

2.2 Superovulations

1. Saline.
2. LHRH ([des-Gly10, D-Ala6]-LH-RH ethylamide acetate salt hydrate) suspended in physiological saline at 0.2 mg/mL.
3. PMSG (pregnant mare serum gonadotropin) suspended in physiological saline at 50 IU/mL (for rats weighing less than 100 g) or 100 IU/mL (for rats weighing more than 100 g).
4. AIS (anti-inhibin antiserum) (Central Research, Tokyo, Japan).
5. hCG (human chorionic gonadotropin) suspended in physiological saline at 50 IU/mL (for rats weighing less than 100 g) or 100 IU/mL (for rats weighing more than 100 g).
6. 1 mL tuberculin syringe with a 26G × 1/2″ needle (Fig. 1a).

2.3 IVF

1. CO_2 gas cylinder.
2. Dissecting scissors (Fig. 1b).
3. Tweezers (Fig. 1b).
4. Sterilized filter paper.
5. 1 mL tuberculin syringe with a 26G × 1/2″ needle (Fig. 1a).
6. 18G × 1/2″ needle (Fig. 1a).
7. HTF.
8. Liquid paraffin.
9. 35 mm culture dish.
10. Three types of mixed anesthetic agents: midazolam (0.4 mg/mL), butorphanol (0.5 mg/mL), and medetomidine (75 μg/mL) adjusted in physiological saline.
11. Mouth pipette.
12. Glass capillary (handmade from a hematocrit tube).
13. Stereoscopic microscope.
14. Warm plate for a microscopic observation.
15. CO_2 incubator.

2.4 Electroporation

1. Alt-R CRISPR-Cas9 crRNA (order made, IDT, IA, USA) suspended in nuclease-free duplex buffer (1072570, IDT) at 200 μM.

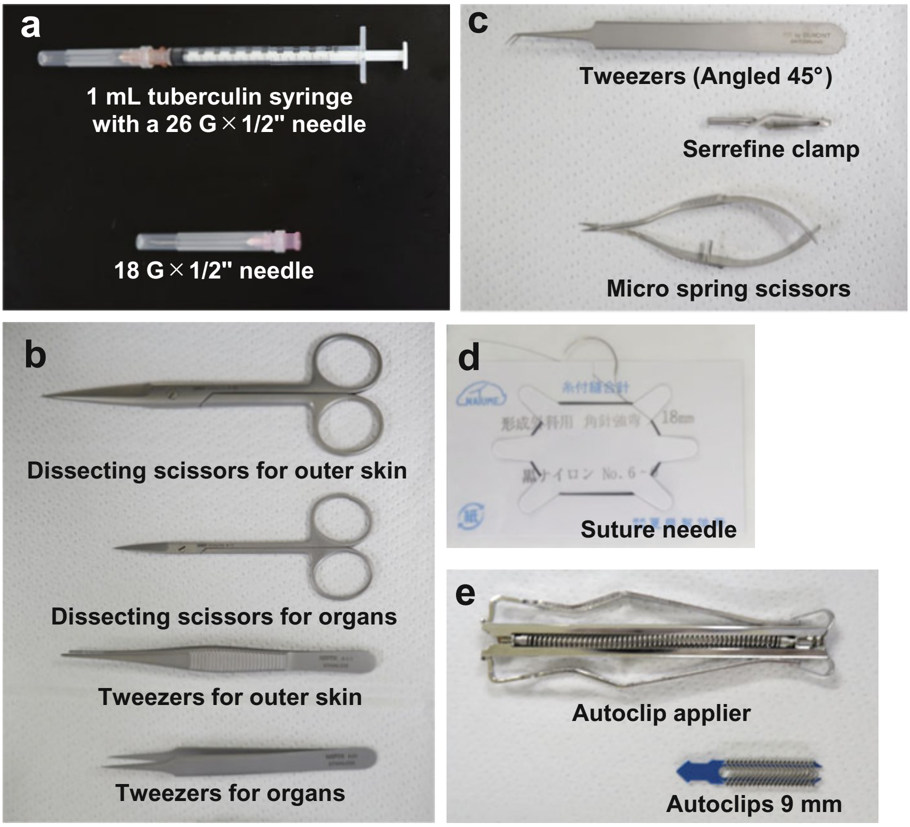

Fig. 1 Surgical instruments. (**a**) Used to administer solutions into the abdominal cavity and IVF. (**b**) Used during IVF and embryo transfer. (**c**–**e**) Used in embryo transfer. These instruments should be used under sterile conditions to prevent infection during surgery

2. Alt-R CRISPR-Cas9 tracrRNA (1072532, IDT) suspended in nuclease-free duplex buffer (1072570, IDT) at 200 μM.
3. Alt-R S.p. Cas9 nuclease V3 (1081058, IDT).
4. Opti-MEM.
5. Thermal cycler.
6. Mouth pipette.
7. Glass capillary (handmade from a hematocrit tube).
8. NEPA21 Super Electroporator (NEPA GENE, Chiba, Japan).
9. Microscope Slide (MS) Platinum Plate Electrodes (CUY505P5, NEPA GENE).
10. Stereoscopic microscope.
11. Warm plate for a microscopic observation.
12. CO_2 incubator.

2.5 Embryo Culture

1. Rat KSOM or HTF.
2. Liquid paraffin.
3. 35 mm culture dish.
4. Mouth pipette.
5. Glass capillary (handmade from a hematocrit tube).
6. Stereoscopic microscope.
7. Warm plate.
8. CO_2 incubator.

2.6 Embryo Transfer

1. Embryo Max Advanced KSOM Embryo Medium (Ad-KSOM) (MR-101-D, Merck, Germany) or PB1.
2. Hair clipper.
3. Dissecting scissors (Fig. 1b).
4. Tweezers (Fig. 1b).
5. Tweezers (angled 45°) (Fig. 1c).
6. Serrefine clamp (Fig. 1c).
7. Micro spring scissors (Fig. 1c).
8. Suture needle (Fig. 1d).
9. Autoclip applier (Fig. 1e).
10. Autoclips 9 mm (Fig. 1e).
11. Three types of mixed anesthetic agents (*see* Subheading 2.3, **item 10**).
12. Atipamezole adjusted in physiological saline at 150 μg/mL.
13. Epinephrine.
14. Mouth pipette.
15. Glass capillary (handmade from a hematocrit tube).
16. Stereoscopic microscope.
17. Warm plate for a microscopic observation.
18. Warm plate for warming rats under anesthesia.
19. Halogen lamp.
20. Bifurcated light.

2.7 Cervical Dislocation

1. CO_2 gas cylinder.
2. Dissecting scissors (Fig. 1b).
3. Tweezers (Fig. 1b).
4. Warm plate for a microscopic observation.

3 Methods

3.1 Superovulation

1. Administer LHRH into the abdominal cavity of juvenile female rats (0.2 mL/rat) between 11:00 am and 5:00 pm.
2. Two days after the administration of LHRH, administer the mixture of PMSG (300 IU/kg) and 0.1 mL of AIS into the abdominal cavity of juvenile female rats between 4:00 pm and 6:00 pm.
3. Approximately 48 h after the administration of the mixture of PMSG and AIS, administer hCG (300 IU/kg) into the abdominal cavity of juvenile female rats between 4:00 pm and 6:00 pm.

3.2 IVF

1. Prepare HTF drops covered with liquid paraffin in culture dishes (one dish/rat for insemination and washing) and equilibrate the culture medium from the evening before performing IVF (Fig. 2a) (*see* **Note 2**).
2. Euthanize a sexually mature male rat by CO_2 and cervical dislocation between 8:00 am and 9:00 am.
3. Dissect the cauda epididymis from the lower part of the testis (Fig. 2b).
4. Clean the fat and blood on the cauda epididymis using a sterile filter paper.
5. Dissect a thicker region of the seminiferous tubule in the cauda epididymis using dissecting scissors (Fig. 2c).
6. Skim spermatozoa from the cauda epididymis using an 18G × 1/2″ needle (Fig. 2d), and transfer the spermatozoa into a 1 mL HTF drop (Fig. 2e).
7. Incubate the sperm suspension for 1 h at 37 °C under 5% CO_2.
8. Add 5 μL of the sperm suspension to 195 μL of TE buffer (pH 8.0) or distilled water in a 1.5 mL tube using a 200 μL pipette with a large opening tip. Mix well by tapping.
9. Count the number of sperms using a cell counter to estimate the number of sperms per mL.
10. Transfer $1.0–2.0 \times 10^5$ sperms to 100 μL of HTF drops (sperm concentration of approximately 1000–2000 sperms/μL) (*see* **Note 3**).
11. Anesthetize superovulated female rats by administering three types of mixed anesthetic agents (0.5 mL/100 g) into the abdominal cavity around 10:00 am (approximately 16–18 h after administrating hCG).
12. Dissect oviducts using dissecting scissors (Fig. 2f). Clean the fat and blood on the oviducts using a sterile filter paper, and transfer the oviducts to liquid paraffin in a culture dish as soon as possible (Fig. 2g).

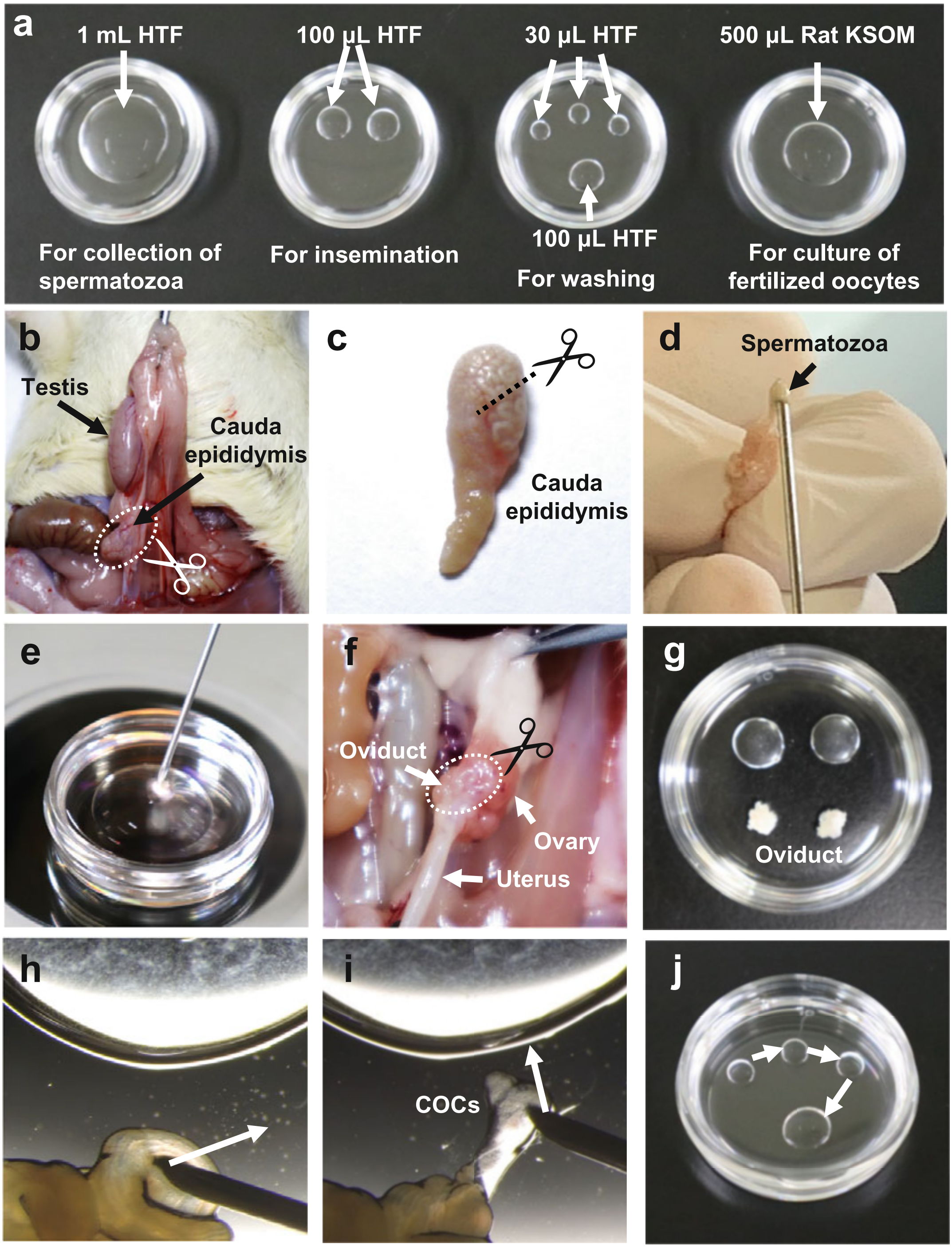

Fig. 2 IVF. (**a**) Preparing each the medium covered with liquid paraffin in culture dish. All medium should be equilibrated in CO_2 incubator. All culture dishes should be handled on a warm plate. (**b**–**e**) Collection of spermatozoa. (**f**–**j**) Collection of oocytes and inseminated oocytes

13. Tear the ampulla of the oviducts by using a 26G × 1/2″ needle and tweezers. Induce cumulus cell-oocyte complexes (COCs) into HTF drops including 1.0–2.0 × 10^5 sperms (Fig. 2h, i).
14. Incubate for 6 h at 37 °C under 5% CO_2 for insemination.
15. After incubation, transfer inseminated oocytes to a 30 μL HTF drops. Wash oocytes by pipetting using a mouth pipette with glass capillary. Repeat washes using 2–3 HTF drops (Fig. 2j) to remove as many sperms and cumulus cells as possible, and then transfer oocytes to a 100–200 μL HTF drop (Fig. 2j).
16. Incubate at 37 °C under 5% CO_2 for 1 h. If possible, select pronuclei-formed oocytes after incubation for 1 h (*see* **Note 4**).
17. During incubation of the washed oocytes, prepare a Rat KSOM drop covered with liquid paraffin in culture dishes (2–4 dishes), and equilibrate at 37 °C under 5% CO_2 for at least 1 h (Fig. 2a).

3.3 Electroporation

1. During incubation of the washed oocytes, prepare 500 μL of Opti-MEM in an IVF dish not covered with liquid paraffin and equilibrate in 37 °C under 5% CO_2 for at least 30 min.
2. Mix 2 μL of Alt-R CRISPR-Cas9 crRNA and 2 μL of Alt-R CRISPR-Cas9 tracrRNA in a PCR tube by pipetting, heat at 95 °C for 5 min using thermal cycler, and place the PCR tube at room temperature.
3. Add 45 μL of Opti-MEM and 1 μL of Alt-R S.p Cas9 nuclease V3 to the PCR tube, mix well by pipetting (final concentration: 8 μM Alt-R CRISPR-Cas9 crRNA, 8 μM Alt-R CRISPR-Cas9 tracrRNA, and 0.2 μg/μL of Alt-R S.p Cas9 nuclease V3), and place the PCR tube at room temperature.
4. Set NEPA21 (poring pulse: voltage (225), pulse width (0.5), pulse interval (50), number of pulse (4), decay rate (10), polarity (+); transfer pulse: voltage (20), pulse width (50), pulse interval (50), number of pulse (5), decay rate (40), polarity (±)), and connect MS platinum plate electrodes to NEPA21) (Fig. 3a) (*see* **Note 5**).
5. Wash a glass chamber by adding 50 μL of Opti-MEM only and pipetting.
6. After removing the Opti-MEM, add 50 μL of the solution containing RNP complexes (*see* **step 3**) to the glass chamber. Remove air bubbles on the surface of two platinum plates by using the tip of a micropipette (*see* **Note 6**).
7. Confirm whether the electrical resistance value is 0.50–0.54 kΩ by pushing the “Ω” button (*see* **Note 7**).
8. Transfer approximately 100 fertilized oocytes into 500 μL of Opti-MEM in an IVF dish.

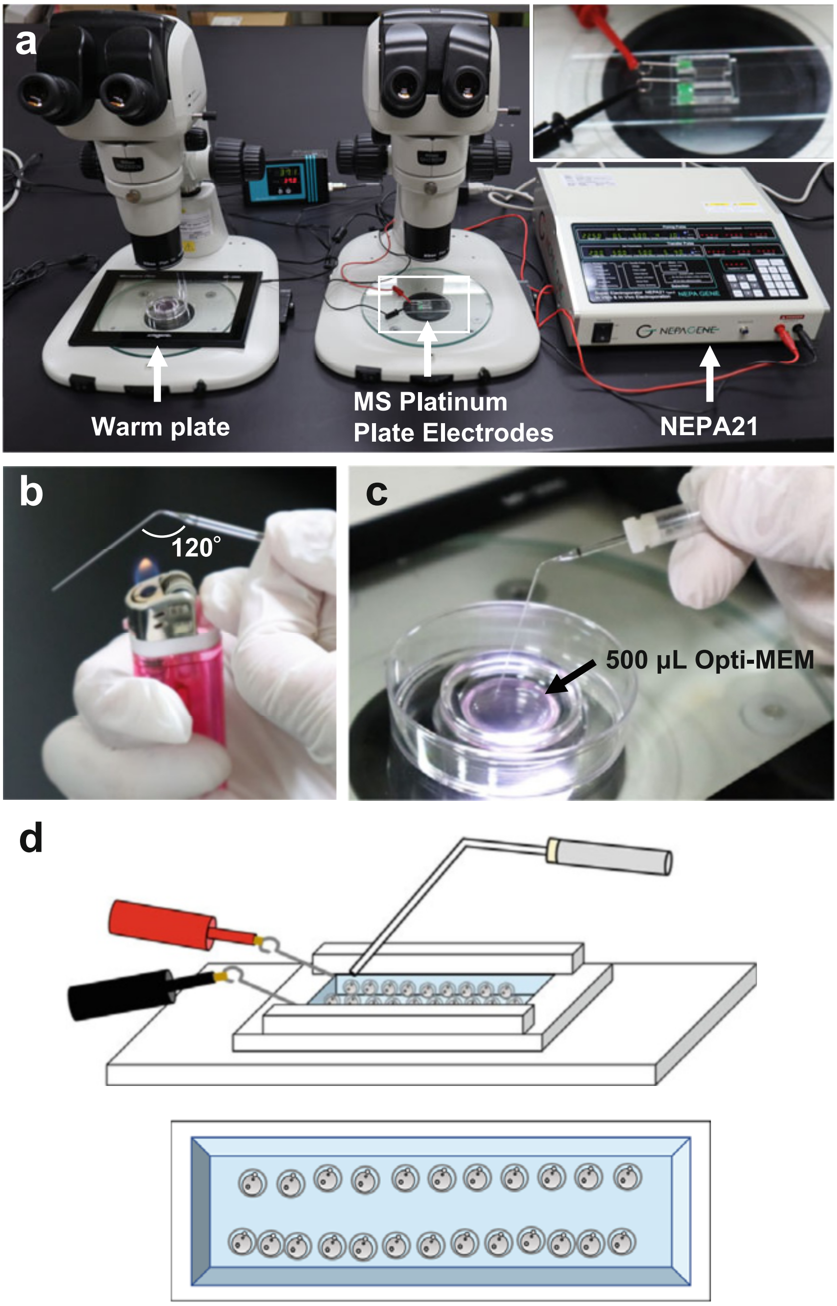

Fig. 3 Electroporation. (**a**) Setup for electroporation. (**b**) Create a bent glass capillary using a lighter. (**c**) Removing liquid paraffin in Opti-MEM. (**d**) Fertilized oocytes lined on a glass chamber

9. Change the glass capillary with liquid paraffin to a new bent glass capillary (Fig. 3b).
10. Recollect all fertilized oocytes while avoiding floating liquid paraffin (Fig. 3c).
11. Place the fertilized oocytes in lines on the glass chamber between the two platinum plates filled with the RNP complex solution (Fig. 3d).
12. Reconfirm the electrical resistance value is 0.50–0.54 kΩ by pushing the "Ω" button (*see* **Note 7**).
13. Push the "Start" button and observe bubbling on the platinum plates under stereoscopic microscopy (*see* **Note 5**).

3.4 Embryo Culture

1. After electroporation, transfer fertilized oocytes into 500 μL of pre-equilibrated Rat KSOM (maximum 50 embryos/500 μL) (*see* **Note 8**).
2. Incubate embryos at 37 °C overnight under 5% CO_2.

3.5 Embryo Transfer

Embryo transfer is performed using a slightly modified version of a previous method [15]. For synchronization of the estrous cycle in female rats, LHRH is used [16, 17]:

1. Five days prior to embryo transfer, administer LHRH into the abdominal cavity of sexually mature female rats (0.2 mL/rat) between 11:00 am and 5:00 pm.
2. One day prior to embryo transfer (the day of IVF), mate sexually mature female rats in proestrus with vasectomized sexually mature male rats to prepare recipients and other sexually mature female rats in proestrus with sexually mature normal male rats to prepare fosters on wire-netting in a cage the evening before transplantation (Fig. 4a) (*see* **Note 9**).
3. The next morning, select female rats with observed plugs (Fig. 4b).
4. Prepare 30 μL of Ad-KSOM drops covered with liquid paraffin in culture dishes (Fig. 4c) and 1 mL of Ad-KSOM in a tube, and warm them for at least 1 h at 37 °C under 5% CO_2.
5. In the afternoon, transfer 30 embryos at one- or two-cell stages to 30 μL of Ad-KSOM drop and separate to each 15 embryos (Fig. 4d) (*see* **Note 10**).
6. Transport the dish including the embryos to a room for embryo transfer.
7. Anesthetize recipient rats by administering three types of mixed anesthetic agents (0.5 mL/100 g) into the abdominal cavity.
8. Shear body hair of recipient rats using hair clippers (Fig. 4e).
9. Dissect a part of the outer skin and the muscle (Fig. 4e).

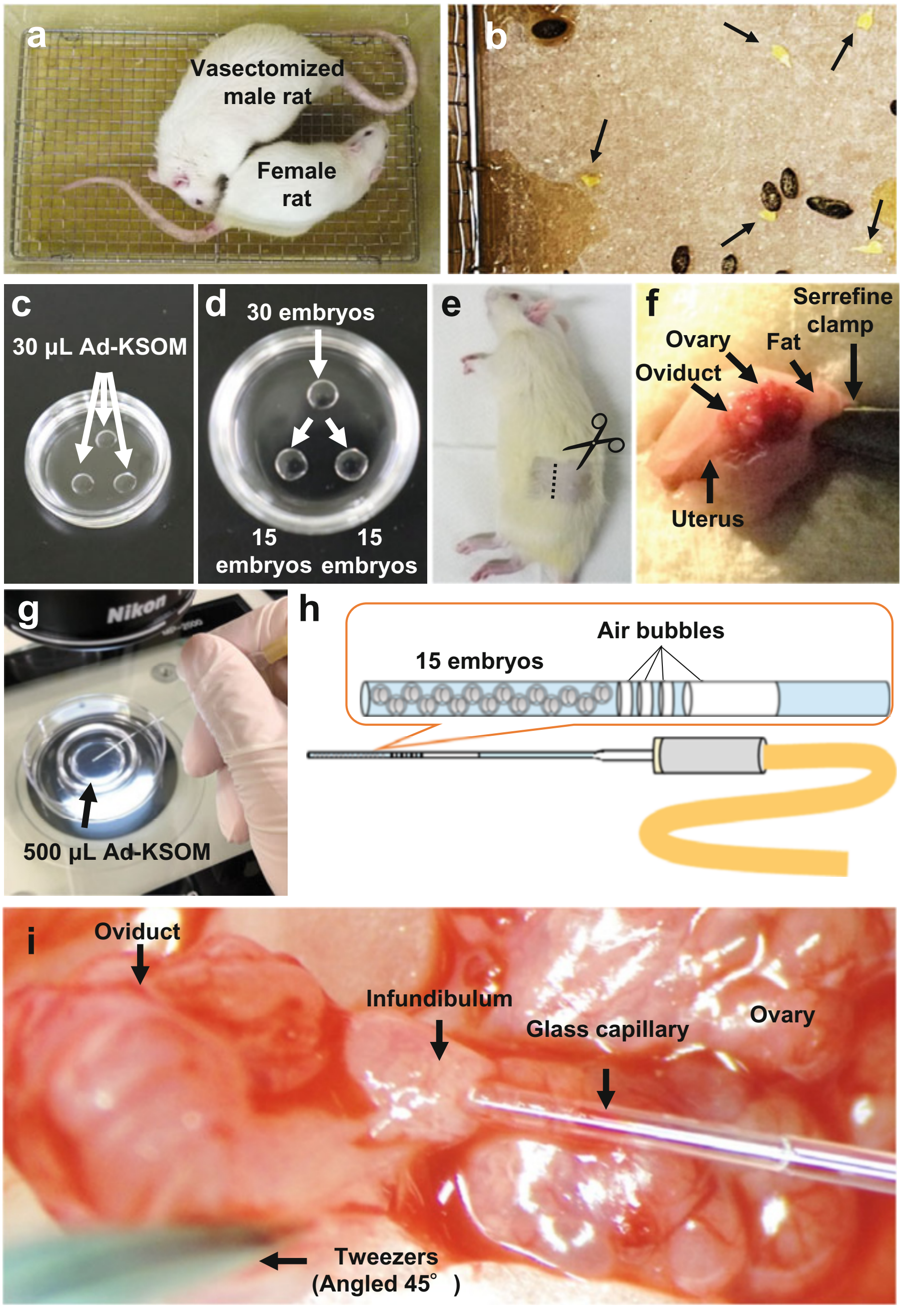

Fig. 4 Embryo transfer. (**a**) Mating vasectomized male rat with female rat. (**b**) Plugs fallen to the bottom of the cage. (**c**) Preparing Ad-KSOM covered with liquid paraffin in a culture dish. (**d**) Transferring embryos to Ad-KSOM. (**e**) Shaving the body hair. (**f**) Exposed ovary-oviduct-uterus out of the body. (**g**) Removing the liquid paraffin in Ad-KSOM. (**h**) Air bubbles and two-cell embryos aspirated in a glass capillary. (**i**) Transferring two-cell embryos into the oviduct

10. Expose the ovary-oviduct-uterus. Secure these organs with serrefine clamping of adjacent fat (Fig. 4f).
11. Drop 10–20 μL of epinephrine onto the ovary to suppress bleeding.
12. Transfer 15 embryos to 500 μL of Ad-KSOM (use the medium warmed at **step 4**) in an IVF dish (Fig. 4g) to remove liquid paraffin with a glass capillary.
13. Aspirate 1 big air bubble, 3 small air bubbles, and 15 embryos into a glass capillary filled with Ad-KSOM (Fig. 4h). Hang the mouth pipette on a stereoscopic microscope.
14. Dissect the ovarian bursa using micro spring scissors and tweezers (Angled 45°), insert the glass capillary into the infundibulum, and transfer the embryos into the oviduct while checking whether three small air bubbles are moving into the oviduct (Fig. 4i).
15. Return the organs into the abdominal cavity, suture the muscle with a suture needle, and close the outer skin with autoclips.
16. Transfer 15 embryos to the other oviduct.
17. Administer atipamezole (same volume to anesthesia) into the abdominal cavity and warm recipients on a warm plate at 37 °C until recovered.

3.6 Cesarean Section

If recipient rats do not give birth 22 days after embryo transfer, perform cesarean section:

1. Euthanize a recipient rat using CO_2 and cervical dislocation.
2. Immediately thereafter, take pups from the uterus using dissecting scissors and tweezers (*see* **Note 11**).
3. Clean off amniotic fluid using a kim wipe and place pups on a kim towel while warming on a hot plate at 37 °C.
4. If a pup does not squeak, gently stimulate the tail using tweezers.
5. Cover pups with woodchip from the cage of the assigned foster for a few minutes to transfer foster odor to the pups.
6. Remove all foster pups and allow the foster to care for the pups delivered by cesarean section.

4 Notes

1. While vasectomized male rats can be purchased, we prepare vasectomized male rats according to previously described methods [17, 18].

2. Culture medium should be equilibrated for at least 1 h before proceeding IVF. After taking the culture dishes from the incubator, place them on a warm plate at 37 °C. Finish the operation and return the culture dishes to the incubator as soon as possible.
3. Typically, 5–10 μL of the sperm suspension is transferred to 100 μL HTF drops.
4. If the selection of pronuclei-formed oocytes is difficult, this procedure is not necessary.
5. Do not warm the glass plate on a warm plate as the RNP complex solution on the glass chamber will be desiccated and the survival rate of fertilized oocytes will be decreased by electroporation.
6. Do not aspirate air bubbles as the volume of RNP complex solution will be decreased. Just poke air bubbles. If air bubbles just float, it is not necessary to completely remove the air bubbles.
7. If the electrical resistance value is lower than 0.50 kΩ, adjust to 0.50–0.54 kΩ by aspirating 2–3 μL of the RNP complex solution at a time. If the electrical resistance value is higher than 0.54 kΩ, adjust to 0.50–0.54 kΩ by adding 2–3 μL of the RNP complex solution or Opti-MEM at a time.
8. HTF can be also used instead of Rat KSOM.
9. As most rats administered with LHRH will be in proestrus after 4 days, smear checks may not be necessary.
10. It is better to use two-cell embryos.
11. Cut the umbilical cord after clamping with tweezers to achieve hemostasis.

Acknowledgments

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Chapter 19

Editing the Genome of the Golden Hamster (*Mesocricetus auratus*)

Michiko Hirose, Toshiko Tomishima, and Atsuo Ogura

Abstract

The golden (Syrian) hamster (*Mesocricetus auratus*) is a small rodent belonging to the Cricetidae family. Golden hamsters have several unique characteristics that are advantageous in the study of reproductive and developmental biology: a highly stable 4-day estrous cycle, a high responsiveness to conventional superovulation methods, and a shortest gestation period (16 days) known among eutherian mammals. Besides these advantages, the technical ease of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in this species has contributed much to our understanding of the basic mechanisms of mammalian fertilization. However, the exceptionally strong in vitro developmental block of hamster embryos, especially at the two-cell stage, has hampered the production of genetically modified hamsters, which has resulted in limited use of this species for biomedical research. However, the recently developed in vivo genome editing method (improved genome editing via oviductal nucleic acid delivery, *i*-GONAD) has overcome this shortcoming and made production of gene-edited hamsters much easier than before. This method has the potential to provide a means of reexamining genes whose functions cannot be identified using mouse models, thus leading to the better understanding of gene functions in mammals. In this chapter, we present our procedure for editing the genome of the golden hamster using *i*-GONAD.

Key words Acrosin, Genome editing, The golden hamster, *i*-GONAD

1 Introduction

Genome editing technology has revolutionized the generation of genetically modified animals. The CRISPR/Cas9 system, in particular, rapidly came into widespread use because gene knockout or knockin animals can be easily and simply generated by designing the appropriate guide RNAs. Thanks to this technology, the trend of generating genetically modified animals, especially mice and rats [1–4], has reached the next peak, leading to the rapid accumulation of information on new gene functions and to the creation of new animal models for biomedical research. However, genome editing

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technology usually requires the in vitro manipulation of zygotes or early-stage embryos, so fundamental reproductive techniques such as collecting fertilized oocytes, embryo culture, and embryo transfer should be easily accessible in the species targeted for such experiments.

The golden (Syrian) hamster (*Mesocricetus auratus*) is a small rodent that has been used as a laboratory species since the early twentieth century [5]. It has several unique characteristics that are advantageous in the study of reproductive and developmental biology: a highly stable 4-day estrous cycle, a high responsiveness to conventional superovulation methods, and a shortest gestation period (16 days) known among eutherian mammals [6, 7]. Furthermore, it has greatly contributed to understanding the mechanism of mammalian fertilization, because standard techniques of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) were first established in this species [8–10]. In addition to these features, it is important to note that the golden hamster is a cricetine rodent, which differs from murid rodents such as the laboratory mouse and rat. The Cricetidae diverged from the Muridae about one million years ago and have evolved differently. Therefore, they are expected to serve as experimental models that are not available for mice and rats. For example, it is known that the lipid metabolism of hamsters is more similar to that of humans than that of mice and rats. Moreover, it has become apparent that hamsters are highly susceptible to new types of coronaviruses and provide good animal models for the study of COVID-19 [11].

However, the golden hamster has a major shortcoming in the use of its oocytes or embryos because the embryos show a very strong developmental block in vitro—especially at the two-cell stage—the so-called two-cell block [12]. Hamster embryos are very sensitive to culture conditions such as temperature fluctuations, low CO_2 levels, visible light, and chemical contaminants in the culture medium. Given this technical difficulty, the production of genetically modified hamsters has been very difficult. As germline-competent embryonic stem cells (ESCs) are not available in hamsters—unlike mice and rats—ESC-mediated gene targeting is also impossible. However, thanks to the advent of the CRISPR/Cas9 system, the first gene knockout hamsters were produced by carefully microinjecting single guide (sg) RNAs and Cas9 into hamster zygotes [13]. As expected, the experiments were not easy to perform because of the extremely high sensitivity of hamster zygotes; therefore, it was necessary to strictly control the time of pronuclear injection and the volume/concentration of sgRNA/Cas9, while avoiding damage to the embryos. Its detailed protocol is now available online and includes movies [14]. With the same strategy, a series of gene knockout hamsters have been produced as models for the study of hypercholesterolemia and atherosclerosis in humans. Because the metabolism of serum lipoproteins differs between humans and mice, the phenotypes of gene knockout mice for factors involved in lipoprotein metabolism have been unclear. By contrast, the similarities in serum lipoprotein metabolism between humans and hamsters have led to the development of excellent models. For example, hamster models of LDL receptor gene knockout [15] and ApoC2 gene knockout [16] have been generated and reported for their utility as disease models.

Thus, the golden hamster has much potential to provide invaluable experimental models that have not been achieved with laboratory mice or rats. However, conventional gene editing strategies for hamsters are available only in a limited number of laboratories worldwide. Therefore, we have focused on the in vivo transfection method (genome editing via oviductal nucleic acid delivery, GONAD) developed by Otsuka et al. at Tokai University [17]. This technology enables genome editing without the need for embryo handling in vitro. However, we found that Cas9 mRNA does not pass through the zona pellucida of hamster zygotes with electroporation pulses, so we employed the *i*-GONAD method that uses the Cas9 protein [18]. First, we applied this technique to elucidate the essential role of the major acrosomal enzyme, acrosin, in fertilization [19] and second to elucidate the role of the PIWI-piRNA system in the oocyte for embryo development by knockout of the *Mov10l1* gene [20]. These issues have not been solved using mouse models because they show no clear phenotypes after gene knockout. It was confirmed that the results of our *Mov10l1* knockout study were consistent with the results of others using the conventional injection method [21, 22]. Therefore, we may expect that continuous development of knockout hamsters with *i*-GONAD will greatly contribute to a broad range of biomedical researches by identifying the functions of genes and providing unique experimental animal models for human diseases. Here, we describe our procedure for genome editing of the golden hamster using *i*-GONAD.

2 Materials

2.1 Preparation of Genome Editing Reagents

1. Cas9 protein.
2. Guide RNAs such as Precision gRNA Synthesis Kit (Invitrogen, cat. no. A29377).
3. CRISPR direct (https://crispr.dbcls.jp/).
4. CRISPOR (http://crispor.tefor.net/).
5. Nuclease-free water.

2.2 Preparation of Hamsters

1. Females: 7–28 weeks of age.
2. Males: 10–40 weeks of age.
3. Anti-inhibin serum (AIS, Central Research Co., Ltd.).
4. Pregnant mare serum gonadotropin.

2.3 Preparation of i-GONAD Experiments

1. Electroporator: Nepa21 (NEPA GENE) (*see* **Note 7**).
2. Electrodes: forceps with Variable Gap 2 Round Platinum Plate electrodes, 5 mm diameter (NEPA GENE, cat. no. CUY650P5).
3. Glass capillary pipettes with filaments such as NARISHIGE, cat. no. GD-1.2.
4. Pipette puller such as P-79/IVF (Sutter Instrument Company).
5. Xylazine.
6. Ketamine.
7. Laboratory wipe paper such as Kimwipe.
8. Phosphate-buffered saline (PBS).

3 Methods

1. Design sgRNAs using CRISPRdirect or CRISPOR (*see* **Note 3**). The number of guides depends on the type of gene knockout, as for mice. The sgRNAs are synthesized using Precision gRNA Synthesis Kit, and the concentration should be 1–3 μg/μl per sgRNA. Nuclease-free water is used (*see* **Note 4**). The genome editing mixture is prepared just before *i*-GONAD experiments. The total volume is 2.5–3.0 μl per oviduct.
2. Preparation of mature female hamsters. Hamsters are kept under controlled lighting conditions (14 h light and 10 h dark, i.e.,, daily light period, 0700–2100) and provided with water and food ad libitum. Females are checked for a regular 4-day estrous cycle. The day of mucous vaginal discharge is designated Day 1. Male hamsters should be exposed to bright light during daytime so that they can mature sexually to enable successful mating [23]. Females are induced to superovulate by subcutaneous injection of 0.25 ml AIS + 25 IU PMSG on the morning of Day 1 (they show a viscous vaginal discharge after estrus). In the evening of Day 4, they are caged with males and left overnight. If they mate successfully, abundant spermatozoa can be observed within the vaginal discharge on the next morning (defined as Day 1 of pregnancy) (*see* **Notes 1–2**).

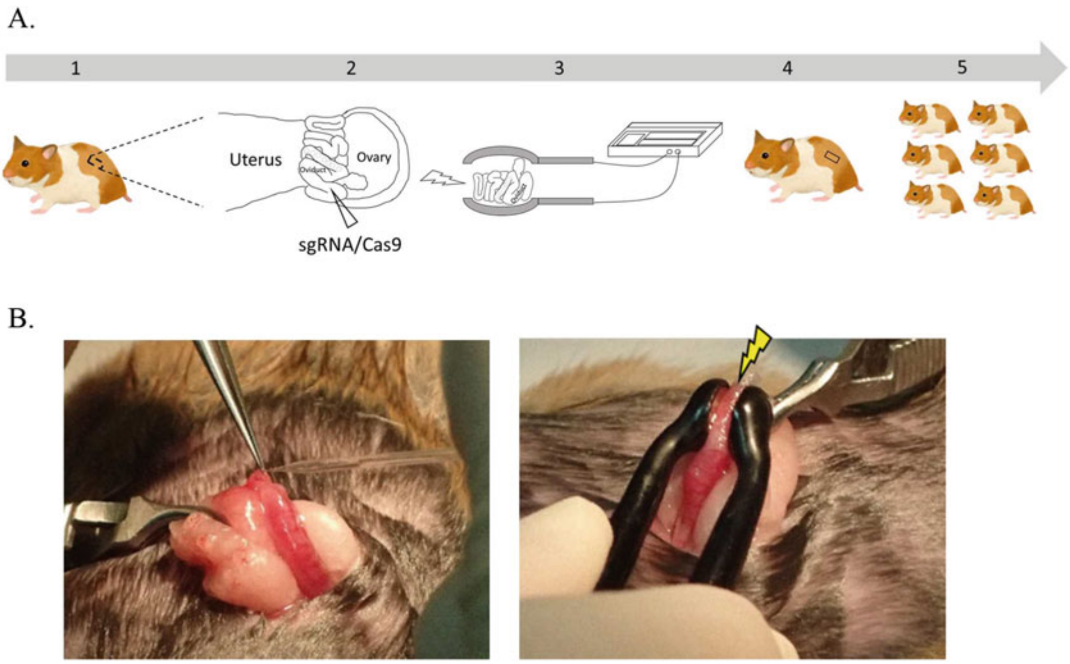

Fig. 1 Experimental design for *i*-GONAD in hamsters. (**a**): Scheme of *i*-GONAD. (**b**): Injection of the sgRNA/Cas9 mixture into the oviduct (left). Electroporation of the embryos in vivo with forceps-like electrodes (right)

3. Anesthesia and surgery. In the evening (19:00–20:00) of Day 1, females are sedated with xylazine (*see* **Note 5**), followed by anesthesia with ketamine (both by intraperitoneal injection). After confirming that the hamsters are anesthetized completely, the ovary and oviduct of one side are exposed through an incision made on the dorsal side.
4. In vivo electroporation (Fig. 1). After confirming the position of the top of the ampulla under the stereomicroscope (*see* **Note 6**), the genome editing mixture (see above) is aspirated into a glass pipette injected into the upper segment of the ampulla. After injection, the oviduct is covered with a piece of PBS-soaked Kimwipe and then pinched with forceps-type electrodes. The distance between the electrodes is about 1.5 mm. Then, electric pulses are applied. The electroporation condition consists of three sequential transfer pulses (400–500 V/cm, 5 ms duration, 50 ms interval) followed by three transfer pulses (100 V/cm, 50 ms duration, 50 ms interval).
5. After the ovary and oviduct are returned to the abdominal cavity, the ovary and oviduct of the other side are exposed and subjected to electroporation as described above.
6. After the incisions have been closed, the hamsters are kept on a warm plate (~37 °C) until they wake up.

7. After surgery, they are placed in a quiet environment and allowed to deliver pups on Day 16 of pregnancy. Unlike mice or rats, Cesarian section delivery is not possible because hamster pups cannot be nursed by lactating foster mothers.
8. For genotyping of the newborn hamsters, ear tissues are collected because hamsters' tails are too short to cut, unlike those of mice (*see* **Note 8**).

4 Notes

1. Unlike female mice, female hamsters show no vaginal plug after mating. So, it is important to check the amount of spermatozoa deposited within the vagina. For this purpose, place a mass of vaginal discharge on a slide glass in the morning following mating and observe the presence/absence of spermatozoa. If the mating is successful, a large amount of sperm mass can be observed by a conventional microscope.
2. There is an individual difference in the ability of males to impregnate females. It is important to record the results of mating for each male so that good males can be consistently used for experiments. Unlike mice, a female and two males can be caged together to increase the chance of pregnancy.
3. Recently, the genome information for the golden hamster has been updated. Please see http://siomilab.keio.ac.jp/hamster.
4. sgRNA can also be prepared by the mixture of crRNA and tracrRNA shortly before experiments, as in mice and rats.
5. Do not use xylazine past their expiration date; it can be highly toxic.
6. As hamster embryos are sensitive to light, it is recommended to cover the oviduct with an aluminum foil sheet as often as possible during operation.
7. As an electroporator, CUY21EDIT II (BEX Co) can be used.
8. For confirmation of the establishment of the *i*-GONAD system for hamsters in your laboratory, we recommend conducting a pilot study, for example, by producing albino hamsters carrying a tyrosinase (*Tyr*) gene mutation (Fig. 2). The gRNA sequence is GGGTGGATGACCGTGAGTCC.

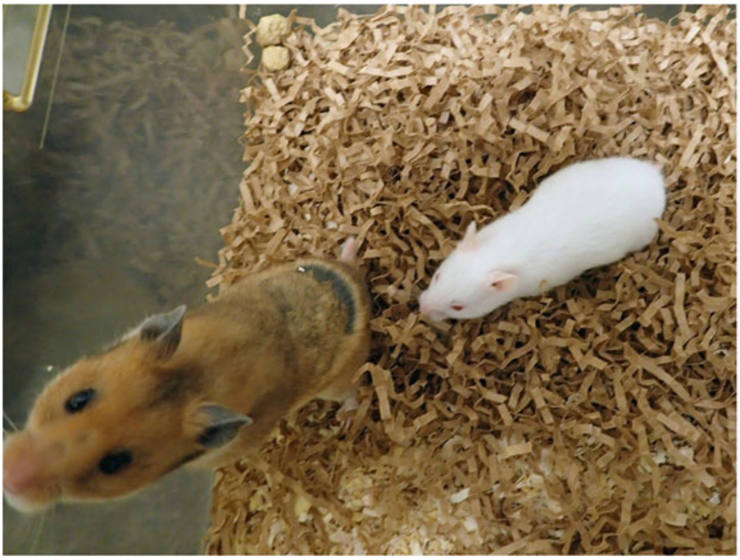

Fig. 2 An albino hamster (right) with its mother (left). It was generated by tyrosinase gene knockout using the *i*-GONAD method

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Chapter 20

Gene Targeting in Rabbits: Single-Step Generation of Knockout Rabbits by Microinjection of CRISPR/Cas9 Plasmids

Arata Honda

Abstract

The development of genome editing technology has allowed gene disruptions to be achieved in various animal species and has been beneficial to many mammals. Gene disruption using pluripotent stem cells is difficult to achieve in rabbits, but thanks to advances in genome editing technology, a number of gene disruptions have been conducted. This chapter describes a simple and easy method for carrying out gene disruptions in rabbits using CRISPR/Cas9 in which the gene to be disrupted is marked, the presence or absence of off-target candidates is checked, and a plasmid allowing simultaneous expression of Cas9 and sgRNA is constructed. Next, the cleaving activity of candidate sequences is investigated, and assessments are carried out to determine whether the target sequences can be cut. Female rabbits subjected to superovulation treatment are mated with male rabbits and fertilized eggs are collected, and then pronuclear injection of plasmid DNA is performed. The next day, the two-cell stage embryos are transplanted into a pseudopregnant rabbits, and offspring are born within approximately 29–30 days. The genomic DNA of the offspring is then examined to check what type of genetic modifications has occurred. With the advent of CRISPR/Cas9, the accessibility of gene disruptions in rabbits has improved remarkably. This chapter summarizes specifically how to carry out gene disruptions in rabbits.

Key words Rabbit, Genome editing, Knockout, Gene targeting, CRISPR/Cas9

1 Introduction

Until the advent of genome editing technology, homologous recombination using mouse embryonic stem (ES) cells and the preparation of chimeric mice were considered mainstream methods for gene disruption in mammals [1]. ES cells from animal species other than mice have an extremely low chimera-forming ability; therefore, performing gene disruption via ES cells was impractical [2]. In addition, if gene disruption is performed via ES cells using species other than mice, ES cells are needed. Furthermore, mice are inexpensive, not subject to certain experimental animal ethics

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restrictions, and excellent in terms of reproduction. Moreover, embryonic manipulation and transplantation techniques are already established. Specifically, one sexually mature rabbit costs about 10,000–30,000 JPY, which is five times higher than the cost of mice and rats but several dozen times lower than the cost of pigs and monkeys; therefore, they can be categorized as relatively inexpensive. Rabbits reach sexual maturity and mate within 4–5 months and have a gestation period of 29–31 days (6–7 pups per birth), and in experiments aimed at creating chimeric animals and producing transgenic animals, they also allow for easy analysis of next-generation influences because of a fast generation cycle and large number of offspring. In addition, as they only weigh approximately 2.5–4.0 kg, a small facility is sufficient to raise them. Rabbits also have a very gentle temperament, and as a result, superovulation injections, as well as anesthetic procedures, can be performed with virtually no resistance if done by an experienced operator. If mating occurs after artificial superovulation, an average of 30–50 fertilized eggs can be collected from each animal. Regarding embryonic transplantations, if 15–20 early-stage embryos are transplanted in oviducts, approximately 30–50% will become offspring, which allows embryonic manipulation and transplantation experiments to be carried out very efficiently. Naturally, the option of using rats could also be put into perspective, but when considering the future application of this technique in humans, we started carrying out gene disruptions in rabbits because the latter is a nonrodent species with a body size larger than that of rats and can be used as a reference when spreading the use of this technique to pigs, nonhuman primates, and large mammals. Homologous gene recombination methods using ES cells are used to be the most common method for carrying out gene disruption in mammals [3], but unlike common ES cells established from mice whose naive state ES cells allow for the formation of chimera and are capable of differentiating into germ cells, ES cells from humans and animal species other than mice are known to be primed state cells with a remarkably poor chimera-forming ability [4]. In other words, because rabbit ES cells are primed state cells, their pluripotent stem cells are extremely difficult to use in the creation of chimera and the induction of differentiation into germ cells [5]. Although research on the conversion of primed state rabbit ES cells into naïve state cells has progressed, true naïve type ES cells that would allow the production of chimeric rabbits have yet to be established [6].

With this background, genome editing technology is very promising, and studies aimed at carrying out gene disruption in rabbits have begun around the world [7–14]. Particularly, the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) (CRISPR/Cas9) system is easy to use and sufficiently simple and efficient to play a leading role in the field of genome editing in rabbits. This chapter describes a genome editing method using CRISPR/Cas9 in rabbits. Particularly, methods aimed at generating gene disruption in rabbits through the pronuclear injection of plasmids are not difficult, even for beginners with little experience in gene recombination. Furthermore, because there is no need to prepare mRNA, this method is highly versatile. This chapter describes both the advantages and disadvantages of the plasmid injection method.

2 Materials

Details pertaining to the construction of plasmids pX330 and pCAG-EGxxFP have been omitted. For more information, please refer to the papers by Mashiko et al. [15] and Honda et al. [10] In addition, gene transfection of plasmids into HEK293 cells can be achieved by standard transfection methods (chemical transfection or electroporation); therefore, details regarding the transfection method have also been omitted.

2.1 Super Ovulation

1. Sexually mature female rabbits for super ovulation (over 4 months old [<2.0 kg]).
2. Sexually mature male rabbits.
3. Porcine follicle-stimulating hormone (pFSH) suspended in physiological saline at 0.5 IU/ml.
4. Human chorionic gonadotropin suspended in physiological saline at 100 IU/ml.
5. Surgical instruments.

   Surgical scissors (B-1H) (Natsume Seisakusho, Tokyo, Japan) (Fig. 1).

   Biological tweezers (A-4) (Natsume Seisakusho) (Fig. 1).
6. Syringes (5 ml, 50 ml).
7. Injection needles (19 and 26 gauge).
8. 100 mm Petri dish.
9. Pentobarbital sodium.
10. Saline.
11. Retention container for rabbit.

2.2 Fertilized Oocyte Recovery and Embryo Culture

1. RD medium: 50% DMEM, 50% RPMI 1640, and 4 mg/ml bovine serum albumin.
2. RD (HEPES) medium: 50% DMEM, 50% HEPES-buffered RPMI 1640, and 0.5% GlutaMAX.
3. Mineral oil.
4. Glass capillaries.

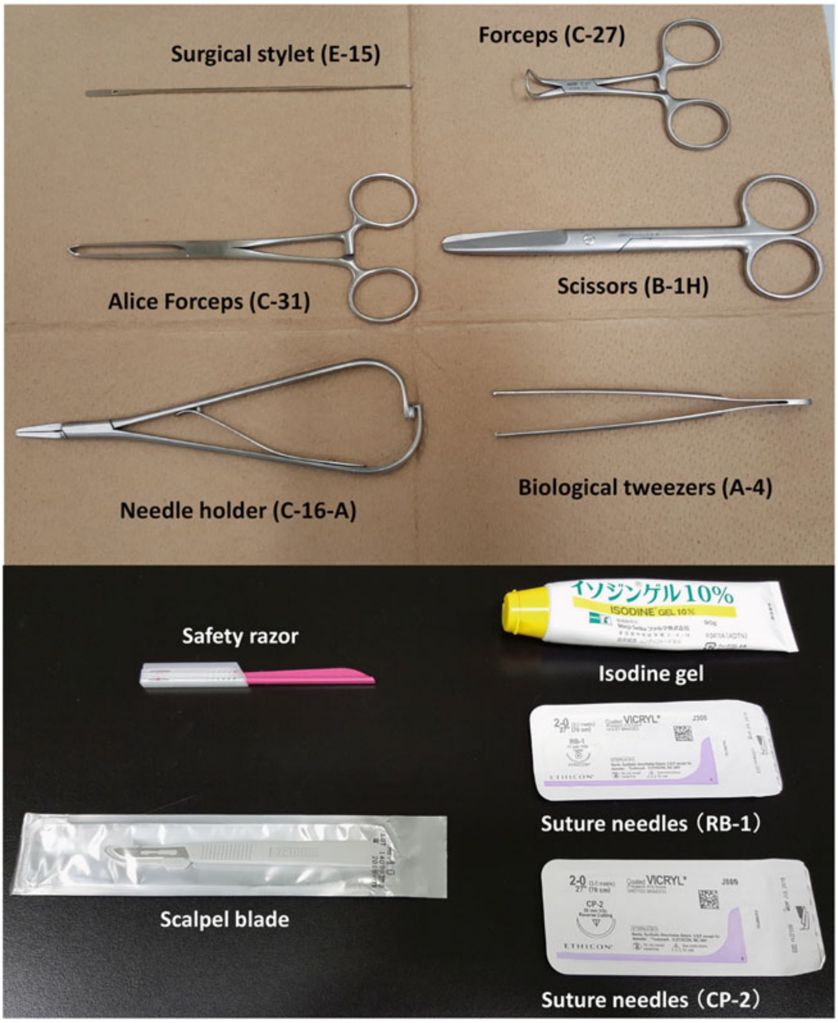

Fig. 1 Surgical instruments. These instruments should be carefully autoclaved and dried to prevent infection during surgery

2.3 Microinjection

1. Phase contrast microscope.
2. Piezo micromanipulator (PMM-150FU) (Prime Tech, Tokyo, Japan).
3. Micropipette puller (P-1000IVF) (Narishige, Tokyo, Japan).
4. Microforge (MF-900) (Narishige).
5. Glass capillary (B100-75-10-PT) (Prime Tech).
6. PVP solution (0.13 g/ml polyvinylpyrrolidone in M2 medium).
7. Plasmids (pX330, pCAG-EGxxFP, pX330-Cetn1, pCAG-EgxxFP-Cetn1) (Addgene, Massachusetts, USA).

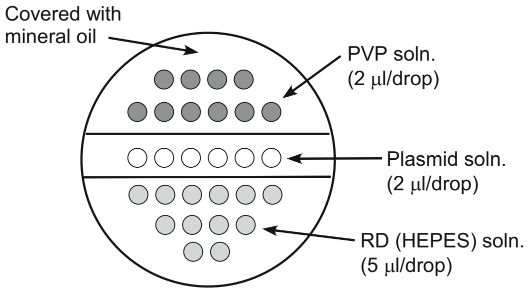

Fig. 2 Injection chamber. The cover of a 100 mm Petri dish filled with appropriate culture medium is used as an injection chamber for injections. RD (HEPES): HEPES-buffered RD medium

8. RD (HEPES) medium.
9. Mineral oil.
10. Cover lid of Petri dish (100 mm) for injection stage (Fig. 2).

2.4 Embryo Transplantation

1. Sexually mature female rabbits for transplantation (foster mothers) (over 4 months old [<2.3 kg]).
2. Surgical instruments (autoclaved) (Natsume Seisakusho).
   Surgical scissors (B-1H) (Fig. 1).
   Biological tweezers (A-4) (Fig. 1).
   Forceps (C27, C31) (Fig. 1).
   Needle holder (C-16A) (Fig. 1).
   Surgical stylet (E-15) (Fig. 1).
3. Glass capillary (handmade from a pasture pipette).
4. RD (HEPES) medium.
5. Insulating container (Cellporter) (Corefront, Tokyo, Japan).
6. Retention container for rabbit.
7. Pentobarbital sodium.
8. Gauze.
9. Surgical cloth.
10. Disinfectant (povidone-iodine, 70% ethanol).
11. 26 gauge needle, 2.5 ml syringe.
12. Safety razor (Fig. 1).
13. Scalpel blade (Fig. 1).
14. Surgical suture needles (Coated VICRYL: RB-1, CP-2 2-0) (Fig. 1).
15. Saline.
16. Surgical gloves.
17. Antibiotics (Cefamezin-α) (Astellas Pharma, Tokyo, Japan).
18. Iodine gel (Meiji Seika Pharma, Tokyo, Japan).
19. Stereomicroscope.

2.5 Parturition and Lactation

1. Nursing cage (W 555 mm × L 605 mm × H 500 mm).
2. Autoclaved floor materials (shredded papers).

3 Methods

3.1 Search for Target Sequences

Investigate the presence or absence of off-target sequences using GGGenome (http://gggenome.dbcls.jp/ja/mm10/2/) and CRISPRdirect (http://crispr.dbcls.jp/). Enter the target sequences (comprising approximately 20 nucleotides), including the PAM (5′-XGG-3′) sequence of the gene that needs to be disrupted (*see* **Note 1**).

3.2 Single-Strand Annealing Assay

1. On the basis that the target sequence has as few off-target candidates as possible, complementary oligo-DNA needs to be ordered from a gene synthesis service and then subjected to an annealing reaction. The target sequence needs to be inserted through ligation into the BbsI site of pX330 (For more details, please refer to Honda et al. [10] and Mashiko et al. [15]).
2. A 500–1000 bp gene fragment containing the target sequence near its center needs to be amplified by PCR and inserted into pCAG-EGxxFP.
3. Prepare plasmids by using the ligation reaction described as above (Subheading 3.2, **steps 1** and **2**) for the transformation of *Escherichia coli* cells, and perform restriction enzyme treatment and sequencing to confirm that the target plasmid has been transformed.
4. Transfect the resulting plasmid into HEK293 cells in the same way as the negative control (pCAG-EGxxFP only) and positive controls (pCAG-EGxxFP-Cetn1 and pX330-Cetn1). Confirm EGFP fluorescence under fluorescent microscopy 24–48 h later. In most cases, if four target sequences are selected, 1–3 targets will exert a cleaving activity equivalent to or stronger than that of Cetn1.

3.3 Superovulation

1. Administer pFSH subcutaneously into the neck of sexually mature female rabbits from 18:00 until 21:00. The pFSH needs to be administered six times in total at intervals of 11–13 h.
2. For rabbits that have completed six administrations of pFSH, approximately 60 h after the beginning of the injections, arrangements need to be made so that rabbits that have been subjected to superovulation treatment are placed in the same

cage as sexually mature male rabbits (female rabbits should be placed in male rabbits' cages) between 19:00 and 21:00 (approximately 72 h after administration of pFSH is initiated) for mating to occur (*see* **Note 2**).

3. Once mating is successful, administer hCG to the female rabbits through the ear vein, and when the bleeding has stopped, place them back in their cage.

3.4 Collection of Fertilized Oocytes

1. Euthanize the rabbits with an overdose of pentobarbital sodium (6 ml per rabbit) 19–20 h after mating.
2. Disinfect the abdomen by spraying with 70% ethanol, and open the abdominal cavity with scissors.
3. Extract the right and left ovaries and fallopian tubes, as well as the uterus, and collect them in a 10 cm Petri dish.
4. Using scissors (B-1H), make an incision near the uterotubal junction perpendicular to the angle of the oviduct. Next, take a 10 ml syringe containing RD (HEPES) (10 ml) culture medium prewarmed at 37 °C, and insert it into the oviduct (which looks like a dot). Then, allow approximately 10 ml of the fluid to flow back into the syringe. The fluid is allowed to flow back while the inserted needle is fixed by forceps outside the oviduct. Transfer the fluid to a 100 mm Petri dish and flush fertilized oocytes from the oviduct into the dish (Fig. 3).
5. Using a stereomicroscope, check the fertilized oocytes (check that the cumulus cells have dispersed), and collect them in a

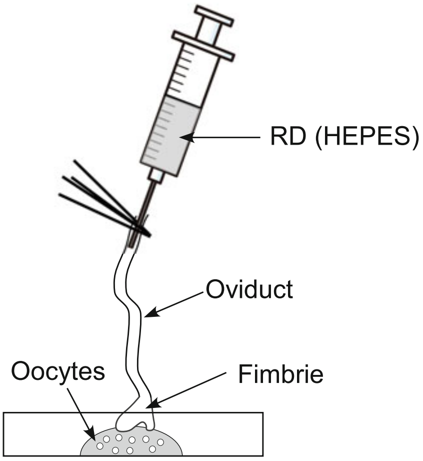

Fig. 3 Oocyte collection. After removing the uterus, ovaries, and most of the fat tissues, insert a round-end needle into the oviduct from the uterus side. The fluid is allowed to flow out while the inserted needle is fixed outside the oviduct using forceps. Fertilized oocytes are then collected in a 100 mm Petri dish. RD (HEPES): HEPES-buffered RD medium

mouth capillary. Next, transfer them in a drop of RD culture medium which is covered with mineral oil and equilibrated in an incubator (37 °C, 5% CO_2 in air). Finally, place them in the incubator.

3.5 Injection of Plasmid DNA

1. Insert the injection pipette into a drop of PVP, suck and eject the PVP liquid two or three times using the manipulator, and then coat the inner surface of the injection pipette with PVP.
2. Take the injection pipette with a PVP-coated inner surface and insert it into a drop of pX330 solution (5–10 ng/μl). Next, fill it with the solution.
3. Remove the fertilized oocytes with pronuclei out of the incubator and transfer them into a drop of RD (HEPES) medium.
4. Hold the oocyte with a holding pipette, insert an injection pipette into the pronucleus, and inject it with pX330 solution. Confirm that the pronucleus is swollen (*see* **Note 3**).
5. After the injection of oocytes is complete, return them to the incubator and culture overnight.

3.6 Embryo Transplantation

1. Foster rabbits are copulatory stimulated by finger press to the vaginal pore 22–26 h before transplantation (Fig. 4).
2. The next morning, select and pick up embryos that have developed into the two-cell stage, and then transfer them into RD

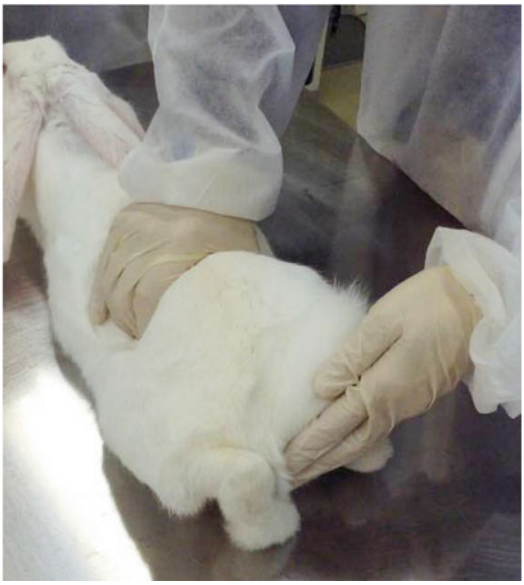

Fig. 4 Preparation of foster mother rabbit for transplantation. Since rabbits ovulate postcopulatory, the foster mother is prepared artificially about 1 day before transplantation

(HEPES) medium. Next, move each dish into a thermal insulation container and take them to the transplantation room.

3. Fill a syringe with 2 ml of pentobarbital sodium and apply 70% ethanol to the ear of the rabbit scheduled for use in transplantation. Tap a blood vessel to make sure it is engorged, and then insert a syringe containing pentobarbital sodium into the blood vessel and administer 1.5 ml of the liquid (*see* **Note 4**).
4. Confirm that the anesthesia is working, and then put the animal on the operating table in the supine position.
5. Place the hind limbs in a way that the knee is maintained in extension in a position that does not block the urethra. Place the forelimbs in extension and immobilize the animal obliquely (using duct tape and rope) on the operating table.
6. Apply a disinfectant (iodine scrub) to the hair at the planned location of the incision, and then wash vigorously and make sure it foams.
7. Confirm the position of the nipples and completely shave the hair using safety razor at the planned location of the incision from top to bottom.
8. Disinfect the skin beforehand with gauze soaked in 1% iodine solution.
9. Cover the animal's entire body with a surgical cloth while exposing only the planned location of the incision, and then pinch the cloth and skin using towel forceps (C-27). Finally, fix with a surgical cloth (*see* **Note 5**).
10. Open a vertical incision of approximately 10 cm in the skin of the midline portion of the abdomen (*see* **Note 6**).
11. Pinch the fascia with forceps (A-4) while making sure that the scalpel blade points upward, and then make a partial incision using the tip of the scalpel (*see* **Note** 7).
12. Pinch the portion of the fascia on the incision line using two pairs of Alice forceps (C-31), and then incise above and under the planned location of the incision using a pair of scissors (B-1H).
13. In addition, pinch the fascia at four locations using two pairs of Alice forceps, and secure the field of view by placing the former laterally to the Alice forceps.
14. In the case that urine has accumulated in the bladder, deflate by sandwiching between both hands and use towels to absorb the urine.
15. Move the appendix and find the uterus before looking for the ovaries and fallopian tubes (either the ones on the right side or those on the left) (*see* **Note 8**).

16. Once the fimbria of the oviduct is found, confirm the capillary insertion location (at the fimbria of the oviduct) and insertion angle using a surgical stylet.
17. Close the incision temporarily (place the Alice forceps crosswise, cover with gauze, and moisten with physiological saline solution to prevent drying).
18. Take the fertilized oocytes and culture fluid out of the carrier box, place them on a thermo-plate, and check them under a microscope.
19. Move the fertilized eggs into the capillary (transplant 5–10 fertilized eggs into an oviduct on one side).
20. Replace the surgical gloves with new ones, open the incision, reexpose the ovaries and fallopian tubes, and reconfirm the capillary insertion site and insertion angle using a surgical stylet.
21. Insert the capillary into the fimbria of the oviduct and transplant the fertilized egg into the oviduct.
22. Unplug the capillary, return the fallopian tube to its original position, and record the number that have been transplanted.
23. Next, search for the oviduct on the opposite side and move it in the same manner as described above.
24. Return the oviducts to their original location, and confirm that no hair is attached to the internal organs (remove any remaining hair with a pair of tweezers).

3.7 Suture: Completion of Surgery

1. Open the bag containing the suture thread (RB-1) and remove with a needle holder.
2. First, suture the center of the surgical wound while gripping the fascia with a pair of tweezers. The suturing has to be performed in 0.5–1 cm intervals using a suturing machine.
3. Open the bag containing CP-2 and take out the suture thread with a needle holder.
4. Let the needle pass through from the edge of the incision to the opposite side of the skin. When performing the suture, take precautions to ensure that the suture thread is not exposed at the surface.
5. Complete the suture by reaching the edges of the wound. Finally, tie a knot using a suturing machine. Make sure that the threads at the stitches and knots are not exposed at the surface (*see* **Note 9**).
6. Inject 1 ml of Cefamezin-α into the thigh muscles.
7. Remove the towel clamps and surgical cloth and apply iodine gel (Fig. 1) to the sutured area.

8. Take the rabbit off the operating table and place it back in its cage.
9. After the rabbit recovers from anesthesia, observe the surgical wound and the animal's general condition closely, and confirm that there are no abnormalities.

3.8 Birth of Offspring

1. The morning after the day of surgery, confirm that the wound is closed and observe the rabbit's condition. Inject Cefamezin-α into the thigh muscles.
2. Approximately 20 days after the surgery, if the rabbit exhibits behavior such as pulling its own hair with its mouth, it may be experiencing a pseudopregnancy.
3. Approximately 24 days after surgery, put a floor cover inside the cage (we use autoclaved and dried paper debris or wastepaper discharged from a shredder). Hug and hold the rabbit as gently as possible to achieve communication and get it acquainted to humans.
4. The birth of the offspring will occur 28–32 days after surgery.
5. It takes approximately 1 month until weaning is complete. The floor cover should be replaced once every 2 weeks. Once weaning is complete, the floor cover can be removed.

4 Notes

1. If possible, the off-target search should be performed on the 15 nt containing a PAM sequence, and the results should show as few off-target sequences as possible. Settings allowing for at least four targets per gene are preferable.
2. Ensure that each female mates with two different males, who should be allowed to ejaculate at least twice. Confirm visually that semen is present in the vicinity of the vaginal pore.
3. Plasmid solution needs to be injected correctly into the pronucleus. If the plasmid is accidentally injected into the cytoplasm, the translation of Cas9 proteins will take a long time, and genome editing is likely to occur after the two-cell embryo stage; this is likely to result in the generation of mosaic embryos.
4. The initial 1 ml should be administered in approximately 5–10 s, whereas the injection of the remaining 0.5 ml should take as much time as possible (30 s or longer) for the effects of the anesthesia to last longer. However, the administration of only pentobarbital sodium is obsolete. Henceforth, using ketamine/xylazine or isoflurane is highly recommended.
5. Be careful not to pinch the nipples with the forceps.

6. Make an incision of about 10 cm starting from a point located at 1 cm below the navel all the way down to the lower abdomen.
7. Point the scalpel blade upward to avoid injuring the intestines.
8. Be careful not to pinch the fallopian tubes with your fingers.
9. If the thread is exposed at the surface, a rabbit that is bothered by the surgical wound may remove it with its mouth or teeth, causing the wound to open.

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Chapter 21

Genome Editing of Pig

Masahito Watanabe and Hiroshi Nagashima

Abstract

Pigs have anatomical and physiological characteristics similar to humans; therefore, genetically modified pigs have the potential to become a valuable bioresource in biomedical research. In fact, considering the increasing need for translational research, pigs are useful for studying intractable diseases, organ transplantation, and regenerative medicine as large-scale experimental animals with excellent potential for extrapolation to humans. With the advent of zinc finger nucleases (ZFNs), breakthroughs in genome editing tools such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) have facilitated the efficient generation of genetically modified pigs. Genome editing has been used in pigs for more than 10 years; now, along with knockout pigs, knock-in pigs are also gaining increasing importance. In this chapter, we describe the establishment of gene-modified cells (nuclear donor cells), which are necessary for gene knockout and production of knock-in pigs via somatic cell nuclear transplantation, as well as the production of gene knockout pigs using a simple cytoplasmic injection method.

Key words Pig, Gene knockout, Knock-in, Somatic cell nuclear transfer, Cloning, Cytoplasmic injection

1 Introduction

The value of pigs as large experimental animals lies in the high potential for extrapolating the findings obtained from pigs to humans. This is because the physiological and anatomical characteristics of pigs are similar to those of humans. Pigs are advantageous in medical research as they are highly fertile and individual production is easy; further, medical equipment and devices used for humans can also be used for pigs without any modification [1]. Rodent models with gene mutations responsible for certain diseases may not reproduce pathological conditions similar to those in humans [2]. In such cases, models have been developed in pigs to reproduce the human pathology rather faithfully [3, 4]. Our models of X-linked severe combined immunodeficiency (XSCID) and autosomal dominant polycystic kidney disease (ADPKD) are good

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examples of this [5, 6]. Thus, development of genetically modified pigs that exhibit pathological conditions closer to humans is important in biomedical research for approaching clinical practice.

In the history of gene modification technologies in pigs, establishment of somatic cell nuclear transfer (SCNT) technology can be considered a major breakthrough [7, 8]. SCNT is a technique used for producing a genetically modified individual with the same genotype as the cell that provides the nucleus, by using cells that have undergone gene modification (gene transfer or knockout) in advance as nuclear donors. The world's first genetically modified cloned pig was a pig from which the sugar chain antigen (αGal), which causes hyperacute rejection in xenografts, had been removed (GGTA1 knockout) [9]. This laid a major foundation for the production of genetically modified pigs. However, as pigs lack germ line transmission-capable embryonic stem (ES) cells [10], and targeted gene modification by homologous recombination using somatic cells was extremely inefficient, production of genetically modified pigs was quite limited until the advent of genome editing. Thus, the inefficiency of genetic engineering technology was one of the hurdles in applying genetically modified pigs to biomedical research. However, the advent of genome editing tools such as zinc finger nucleases (ZFNs) [11], transcription activator-like effector nucleases (TALENs) [12, 13], and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) [14, 15] has accelerated genetic modification in pigs [16–20]. Currently, genome editing technology has been used to develop several medical research pigs, including disease model pigs [5, 6, 21], xenograft donor pigs [18, 22], and organ-deficient pigs for use in organ regeneration [23–25]. Furthermore, it is being used for producing genome-edited pigs for application to agriculture (livestock) [26–28].

In addition to SCNT, cytoplasmic injection and electroporation of zygotes [29] are used as methods for producing gene knockout pigs using genome editing technology (Fig. 1). The cytoplasmic injection and zygote electroporation methods are simple; however, whether or not the desired genotype is reliably obtained can be clarified only after the individual is actually produced. Further, the occurrence of mosaic (a state in which cells with multiple different genotypes coexist in one individual) remains a problem to be solved [30–32]. On the contrary, in the SCNT method, subjecting the nuclear donor cell to the desired gene modification in advance allows reliable production of an individual with only the desired genotype. Another advantage is that SCNT does not produce mosaic individuals.

More than 10 years have passed since genome editing was first used in pigs, and now, knockout pigs can be created very easily. Subsequently, knock-in, which inserts a target DNA sequence into a specific locus of interest, has recently been increasingly applied to

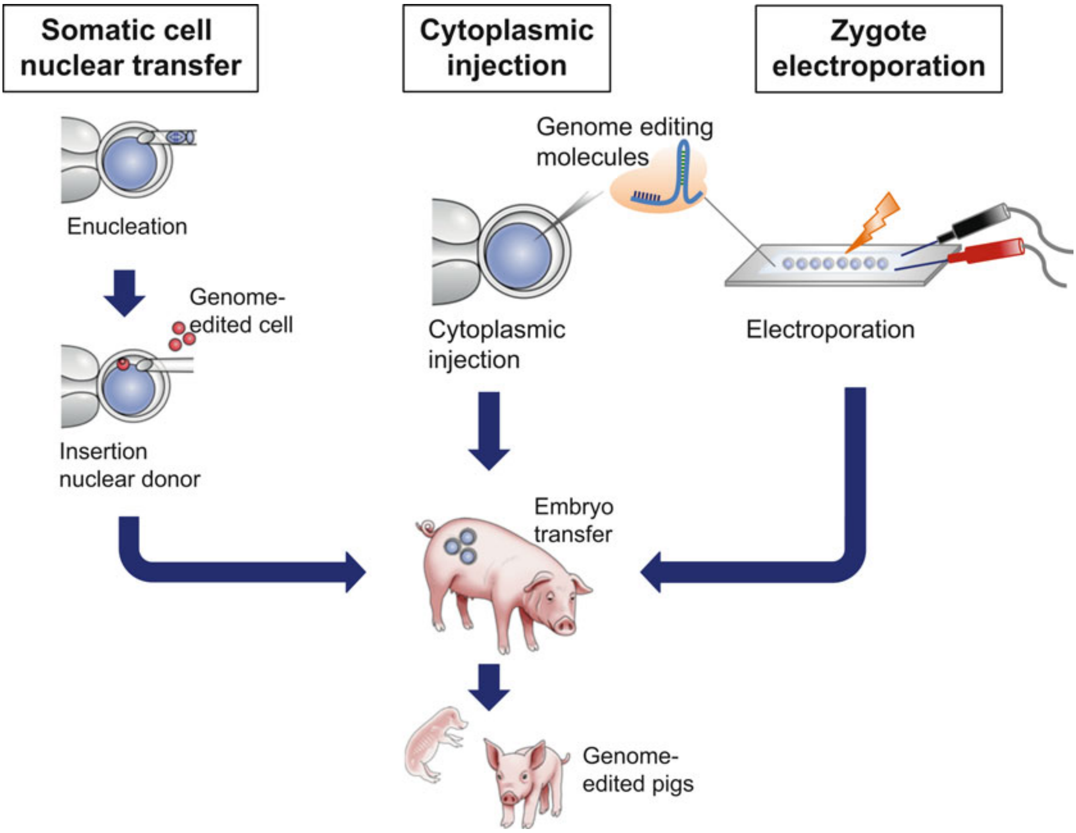

Fig. 1 Schematic representation of strategies for generating genetically modified pigs by genome editing

pigs. SCNT is currently the most robust method for producing gene knock-in pigs, which require precise gene modification. In fact, most knock-in pigs are produced by SCNT using genetically edited somatic cells (mainly fibroblasts) [33–35].

The production of knock-in pigs using cytoplasmic injection is also inefficient, with limited reports in pigs. Regarding knock-in generation using the zygote electroporation method, unlike mouse eggs, pig eggs are highly sensitive to electricity, which impedes the knock-in of large molecules containing transgenes; therefore, future development is awaited in this regard [36].

In this chapter, we introduce the production of genetically modified pigs using two approaches, SCNT and cytoplasmic injection. For technical details on individual production by SCNT, see the appendix of this series [37], which focuses on the establishment of gene knockout and knock-in cells that serve as nuclear donors.

2 Materials

2.1 Preparation of Genome Editing Tools

2.1.1 ZFNs

1. CompoZr Custom Zinc-finger nuclease (Sigma-Aldrich).

2.1.2 TALENs

1. Custom TALEN synthesis service (Thermo Fisher Scientific).
2. Platinum Gate TALEN Kit (Addgene).

2.1.3 CRISPR/Cas9

1. pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene).
2. crRNA.
3. tracrRNA.
4. Cas9 nuclease protein.

2.1.4 In Vitro Transcription

1. In vitro transcription kit such as mMESSAGE mMACHINE T7 Ultra Transcription Kit (Thermo Fisher Scientific).
2. In vitro transcription kit such as MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific).
3. DNase-/RNase-free distilled water.
4. RNase decontamination reagent: for eliminating of RNase contamination.
5. Plasmid extraction kit such as EndoFree Plasmid Maxi Kit (QIAGEN).
6. Isopropanol.
7. Restriction enzyme.
8. 99.5% ethanol.
9. TE saturated phenol: Saturated with 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA.
10. Chloroform.
11. 3 M sodium acetate (pH 5.2).

2.1.5 Purification of RNA

1. RNA cleanup kit such as MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific).

2.1.6 Nuclease-Based Mutation Detection Assay

1. Genomic DNA extraction kit such as DNeasy Blood and Tissue Kit (QIAGEN).
2. Mutation detection kit such as Guide-it Mutation Detection Kit (Takara Bio).

2.2 Isolation of Nuclear Donor Cells for SCNT

2.2.1 Preparation for Porcine Fetal Fibroblasts (PFFs) for Gene Knockout

1. Antibiotic-antimycotic solution.
2. Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 2% (v/v) antibiotic-antimycotic solution.
3. Iris scissors and forceps.
4. Collagen type I coated 75 cm^2 culture flask (AGC Techno Glass).
5. Stainless steel sieve.
6. Cryogenic vial.
7. Cellbanker (Takara Bio): for cryopreservation of PFFs.
8. 100 mm plastic Petri dish.
9. Cell strainer.
10. Syringe plunger.
11. 0.05% Trypsin-EDTA.
12. Insulin-Transferrin-Selenium-Ethanolamine (ITS-X).
13. MEM Non-Essential Amino Acid Solution (NEAA).
14. Culture medium: Minimum Essential Media (MEM)α (Thermo Fisher Scientific) supplemented with 15% (v/v) fetal bovine serum (FBS), 2% (v/v) antibiotic-antimycotic solution, 1% (v/v) ITS-X, and 1% (v/v) NEAA.

2.2.2 Electroporation

1. Neon Transfection System (Thermo Fisher Scientific).
2. Dulbecco's phosphate-buffered saline (PBS, DNase-RNase-free grade).
3. Culture medium: MEMα supplemented with heat-inactivated 15% FBS with or without 1% (v/v) antibiotic-antimycotic solution.

2.2.3 Preparation of Knock-in Donor Template or Vector

1. Directional cloning kit such as In-Fusion HD Cloning Kit (Takara Bio).
2. Direct PCR enzyme such as MightyAmp DNA Polymerase Ver.2 (Takara Bio).
3. PCR enzyme such as PrimeSTAR GXL DNA polymerase (Takara Bio).
4. BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific).
5. ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific).
6. PCR Cloning Kit such as Zero Blunt TOPO PCR Cloning Kit for Sequencing (Thermo Fisher Scientific).
7. Cloning vector such as pBluescript II Vector (Agilent).

8. LB agar plate including 50 μg/mL of ampicillin or kanamycin.
9. Plasmid extraction kit such as Endotoxin-free Plasmid DNA isolation kit (QIAGEN).
10. Custom single-stranded oligodeoxynucleotides (ssODN).

2.2.4 Isolation of Genetically Modified Cells (Nuclear Donor)

1. MEMα (Thermo Fisher Scientific).
2. Fetal bovine serum (FBS).
3. Antibiotic-antimycotic solution.
4. 0.05% Trypsin-EDTA.
5. PBS (−).
6. Collagen type I coated 60 and 100 mm dish (AGC Techno Glass).
7. Collagen type I coated 96-, 48-, 24-, 12-, and 6-well plate (AGC Techno Glass).
8. Cell counter such as Countess Automated Cell Counter (Thermo Fisher Scientific).
9. Countess Cell Counting Chamber Slides (Thermo Fisher Scientific).
10. Cryovial tube.
11. Cellbanker.
12. Culture medium: for culture of PFFs. MEMα supplemented with heat-inactivated 15% FBS and 1% (v/v) antibiotic-antimycotic solution.
13. Cell strainer.
14. Multichannel pipette.
15. Pipetting reservoir.
16. Direct PCR enzyme such as MightyAmp DNA polymerase Ver.2 (Takara Bio).
17. PCR enzyme such as PrimeSTAR HS DNA polymerase (Takara Bio).
18. BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific).
19. ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific).
20. PCR cloning kit such as Zero Blunt TOPO PCR Cloning Kit for Sequencing (Thermo Fisher Scientific).
21. LB agar plate including 50 μg/mL of ampicillin or kanamycin.

2.3 Somatic Cell Nuclear Transfer (SCNT)

2.3.1 Preparation of In Vitro Matured (IVM) Oocytes

1. PBS-PVA: PBS containing 75 μg/mL potassium penicillin G, 50 μg/mL streptomycin sulfate, and 0.1% (w/v) polyvinyl alcohol (PVA).
2. Hexadecyltrimethylammonium bromide (CETAB) stock: Dissolve 25 g of CETAB in 125 mL 99.5% ethanol and bring the volume up to 500 mL with water.
3. CETAB solution: Dilute CETAB stock to 0.2% solution with water.
4. Porcine oocyte/embryo collection medium (POE-CM, Research Institute for the Functional Peptides): for oocyte collection.
5. North Carolina State University (NCSU)-23 medium: 108.73 mM NaCl, 4.78 mM KCl, 1.7 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 5.55 mM glucose, 7.0 mM taurine, 50 mg/L streptomycin sulfate, and 65 mg/L penicillin-G in water.
6. High-performance porcine oocyte medium (HP-POM; Research Institute for the Functional Peptides).
7. Dibutyryl cyclic adenosine monophosphate (dbcAMP, Research Institute for the Functional Peptides).
8. Porcine follicular fluid: collect follicular fluid from ovaries (3–6 mm of follicles in diameter) by aspiration and centrifuged at 1200 × *g* for 60 min at 4 °C.
9. NCSU-based maturation medium (for SCNT): NCSU23 supplemented with 10% (v/v) porcine follicular fluid, 10 IU/mL equine chorionic gonadotropin (eCG), and 10 IU/mL human chorionic gonadotropin (hCG), 0.6 mM cysteine, 10 ng/mL EGF, 1 mM L-glutamine, and 5 mM hypotaurine.
10. POM-based maturation medium (for in vitro fertilization): HP-POM supplemented with 10% (v/v) porcine follicular fluid, 10 IU/mL equine chorionic gonadotropin (eCG) and 10 IU/mL human chorionic gonadotropin (hCG), and 1 mM dbcAMP.
11. Paraffin oil.
12. 35 and 60 mm plastic Petri dish.
13. Thermo-container.
14. Needles (20G).
15. Syringes (10 mL).
16. Warm plate (38.5 °C).

2.3.2 Nuclear Transfer

1. MEMα: for culture of nuclear donor.
2. FBS.

3. HEPES-buffered Tyrode's lactose medium with 0.3%(w/v) PVP (HEPES-TL-PVP): 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone in Tyrode lactose medium.
4. Hyaluronidase: 1 mg/mL solution in HEPES-TL-PVP.
5. Demecolcine solution (Sigma-Aldrich): 10 μg/mL in Hank's Balanced Salt Solution (HBSS).
6. Cytochalasin B stock: 5 mg/mL solution in dimethyl sulfoxide (DMSO).
7. Fusion medium: 280 mM mannitol, 0.5 mM HEPES, 0.15 mM $MgSO_4$, and 0.01% (w/v) PVA in water.
8. Electrode needles (Nepa Gene).
9. Somatic hybridizer (Nepa Gene).
10. Porcine zygote medium-5 (PZM-5, Research Institute for Functional Peptides): for in vitro culture.
11. PZM-5 (BSA) (Research Institute for the Functional Peptides): PZM-5 without PVA supplemented with 4 mg/mL BSA.
12. Fusion chamber slide (MS Platinum Wire Electrode 1 mm gap, Nepa Gene).
13. Activation solution: 280 mM mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) PVA.
14. Electrical pulsing machine (Multiporator, Eppendorf).
15. Scriptaid stock: 2.5 mM Scriptaid in DMSO.

2.4 Cytoplasmic Injection

2.4.1 In Vitro Fertilization (IVF) of Porcine Oocytes

1. PBS-BSA: PBS supplemented with 0.1% bovine serum albumin (BSA).
2. Porcine fertilization medium (PFM; Research Institute for the Functional Peptides).
3. HEPES-buffered Tyrode's lactose medium with 0.3% (w/v) PVP (HEPES-TL-PVP): 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone in Tyrode lactose medium.
4. HEPES-TL-PVP supplemented with 10% FBS: for checking the first polar body.
5. PZM-5.
6. PZM-5 (BSA).
7. Paraffin oil.
8. 35 mm plastic Petri dish.

2.4.2 Cytoplasmic Injection to Porcine Zygotes

1. Micromanipulation system.
2. Microinjector.
3. Microloader tip.

4. Injection pipette: the injection pipettes are made with a micropipette puller (Sutter instrument) with the following adjustments: heat, 675; velocity, 30; and time, 250.
5. Capillary glass for holding pipette: diameter is 150 μm.
6. Inverted microscope.
7. DNase-/RNase-free water: for dilution of RNAs.
8. TE^{-1} buffer: 10 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA (pH 8.0).
9. HEPES-TL-PVP supplemented with 5% FBS: HEPES-TL-PVP containing 5% FBS; adjust osmotic pressure 290 mOsm.
10. PZM-5.
11. Mineral oil.
12. Paraffin oil.
13. 35 mm plastic Petri dish.

2.5 General Equipment and Plastics

1. Humidified incubator at 30 °C and 37 °C and 5% CO_2 for cell culture.
2. Humidified incubator at 38.5 °C and 5% CO_2 for embryo culture.
3. Humidified incubator at 38.5 °C and 5% CO_2, 5% O_2, and 90% N_2 for embryo culture.
4. Water bath (37 °C, 55 °C).
5. Freezers (−30 °C, −80 °C) and refrigerators (4 °C).
6. Phase contrast microscope.
7. Stereoscopic microscope.
8. Pipetman (2, 10, 20, 200, and 1000 μL).
9. Tips for 2, 10, 20, 200, and 1000 μL pipetman.
10. Plastic tube (1.5 mL).
11. Conical tube (15 mL, 50 mL).
12. Thermal cycler.
13. Electrophoresis system.
14. 0.2 mL PCR tube and 96-well PCR plate.
15. Spectrophotometer.
16. Liquid nitrogen storage tank.
17. Plastic pipettes (1, 5, 10, and 25 mL).
18. Centrifuge.
19. Clean bench and biosafety cabinet.
20. 37 °C shaker (at 160–200 rpm).

3 Methods

3.1 Preparation of Genome Editing Tools

Different protocols are available (*see* Chapters 1, 2, and 3). All steps require DNase-/RNase-free reagents and equipment such as microcentrifuge tubes and pipette tips for electroporation or cytoplasmic injection of RNAs.

3.1.1 ZFNs

Basic handling and design/construction for ZFNs are described in Chapter 1 (*see* **Note 1**).

3.1.2 TALENs

The basic handling and design/construction of TALENs are described in Chapter 2 (*see* **Note 2**).

3.1.3 CRISPR/Cas9

The basic handling and design/construction of CRISPR-Cas9 are described in Chapter 3 (*see* **Note 3**).

3.1.4 In Vitro Transcription

Use the mMESSAGE mMACHINE T7 Ultra Transcription Kit for ZFN-, TALEN-, and Cas9-mRNAs. For in vitro transcription of guide RNA (gRNA), use the MEGAshortscript T7 transcription Kit in this step. These protocols essentially correspond to the manufacturer's instructions (*see* **Note 4**).

3.1.5 Purification of RNA

Use the MEGAClear Kit for this step. This protocol essentially corresponds to the manufacturer's instructions (*see* **Note 5**).

3.1.6 Nuclease-Based Mutation Detection Assay (Cleavage Detection Assay)

The cleavage detection assay with mismatch-specific nucleases is useful for validating the cleavage efficiency of CRISPR-Cas9 (*see* **Note 6**). Use the Guide-it Mutation Detection Kit for this step. This protocol essentially corresponds to the manufacturer's instructions.

3.1.7 Construction of Donor Vector for Knock-in

Donor plasmid vectors for gene knock-in are constructed using In-Fusion Cloning, which allow ligation-independent directional cloning, with the pBluescript II plasmid vector as the backbone (*see* **Note 7**).

3.2 Isolation of Nuclear Donor Cells for SCNT

When isolating nuclear donor cells, plasmid DNA is commonly used for ZFNs, TALENs, or CRISPR-Cas9 expression; however, we used RNAs encoding these to prevent foreign genes from being inserted into the individual genomes of animals. Further, Cas9 protein can be used instead of Cas9-mRNA in the CRISPR-Cas9 system. Although various techniques for gene knock-in to cells are now available, traditional gene targeting using donor vector combined with genome editing tools in pig fibroblasts is used for generating knock-in pigs via SCNT.

3.2.1 Preparation for PFFs for Genetically Modified Cells

Generally, a primary culture of porcine fetal fibroblasts (PFFs) with high growth performance is suitable as the progenitor line for isolating genetically modified cells (*see* **Note 8**).

1. The uterus containing fetuses is excised surgically from pregnant sow under anesthesia (*see* **Note 9**).
2. Fetuses are recovered from the uterus, sacrificed, and transferred to a plastic dish on ice.
3. In the clean bench, the head and visceral tissue of each fetus are removed using iris scissors and forceps and then washed three times using PBS supplemented with antibiotic-antimycotic.
4. The remnants are minced with scissors on a 100 mm Petri dish, followed by grinding with a syringe plunger and stainless steel sieve to eliminate clumps and debris.
5. Rinse the stainless steel sieve with culture medium; the pass-through sample is collected as dispersed cell suspensions.
6. Seed the cell suspension into 75 cm^2 collagen-coated flasks.
7. When the cells reach the subconfluent stage (70–80% confluency) in the flask, the cells are harvested after treatment with 0.05% Trypsin-EDTA and passaged to a new flask for the further propagation (*see* **Note 10**).
8. Under subconfluent conditions, the PFFs are harvested and cryopreserved with Cellbanker at a concentration of 5×10^5 cells/tube for later use (*see* **Note 11**).

3.2.2 Electroporation

Use the Neon Transfection System for this step. Electroporation is conducted essentially according to manufacturer's instructions.

1. Low passaged PFFs (P1–P3, Subheading 3.2.1) are seeded to be 70–90% confluent at the time of electroporation on a 100 mm collagen-coated dish.
2. On the day of electroporation, PFFs are washed twice with PBS and harvested after treatment with 0.05% Trypsin-EDTA.
3. After adding the culture medium to stop the trypsin reaction, harvest and centrifuge at 150 × *g* for 5 min at room temperature.
4. Carefully remove the liquid; the cell pellets are suspended with 1 mL of DNase-/RNase-free PBS (*see* **Note 12**).
5. After cell counting, 5×10^5 cells are transferred to a 1.5 mL tube and centrifuged at 150 × *g* for 5 min at room temperature.
6. Carefully remove the liquid and resuspend the PFFs in 50 μL (1×10^5 cells/10 μL) of R-buffer (included in the Neon Transfection system).

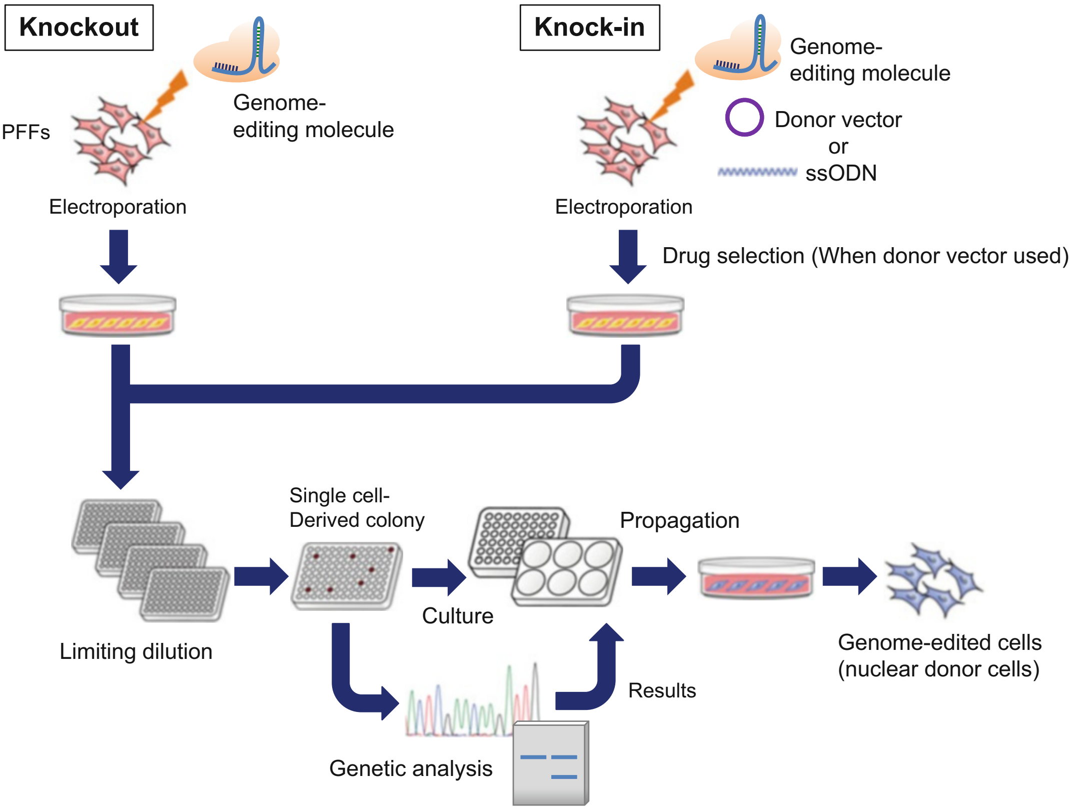

Fig. 2 Schematic representation of isolation for genome-edited cells (gene knockout or knock-in-nuclear donor) for somatic cell nuclear transfer (SCNT)

7. Add ZFNs, TALENs, or CRISPR-Cas9 (and donor vector or ssODN for knock-in only) to the cell suspension and mix gently (*see* **Note 13**).
8. Electroporation is performed using the Neon Transfection System with 10 μL Neon tip (*see* **Note 14**).
9. After electroporation is complete, transfer the electroporated cells (5 × 10^5 cells) to a 100 mm collagen-coated dish containing 10 mL of pre-warmed culture medium without antibiotic-antimycotic (*see* **Note 15**).

3.2.3 Isolation of Gene Knockout Cells (Nuclear Donor)

An overview of this step is presented in Fig. 2.

1. After electroporation (Subheading 3.2.2), the ZFN- or TALEN-treated cells are cultured at 30 °C for 72 h (*see* **Note 16**). For CRISPR-Cas9, the treated cells are cultured continuously at 37 °C.
2. When the cells reach the sub-confluent stage (generally after 48 or 72 h of electroporation), the cells are harvested for limiting dilution to single cell cloning.

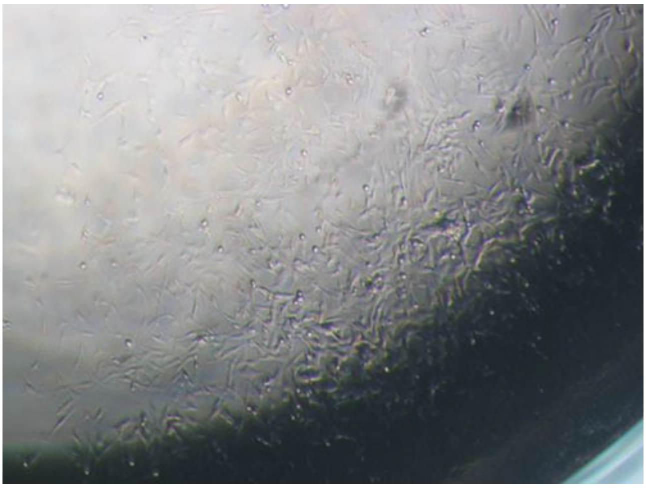

Fig. 3 Single cell-derived colony with high proliferative performance in a 96-well plate after eight days of limiting dilution

3. Count the number of live cells and dilute them serially with culture medium at a concentration of 5 cells/mL (*see* **Note 17**).
4. Dispense one cell/well (200 μL) in 96-well plates using a multichannel pipette, and transfer the plates to a humidified incubator at 37 °C.
5. Change the half volume of culture medium (100 μL) every 3 days (*see* **Note 18**).
6. After 7–8 days of culture, check the colony formation derived from a single cell (*see* **Note 19** and Fig. 3).
7. At 12–14 days after limiting dilution, cells at relatively high confluency (>50%) with good morphology in each well are selected and divided for further culture (transferred to a 48-well plate) and mutation analysis (*see* **Note 20**).
8. For mutation analysis, direct PCR from the divided cells in the well is performed to amplify the target sequence and confirm the ZFN-, TALEN-, or CRISPR-Cas9-induced mutations by direct sequencing (*see* **Note 21**).
9. In parallel with mutation analysis (**step 8**), the cell culture is expanded (*see* **Note 22**).
10. Finally, harvest single cell-derived clones with gene knockout, suspend the cells in Cellbanker, and cryopreserve as nuclear donor cells at −80 °C or in liquid nitrogen until SCNT (*see* **Note 23**).

3.2.4 Isolation of Gene Knock-in Cells (Nuclear Donor)

An overview of this step is presented in Fig. 2. The steps for isolating knock-in cells are similar to those for knockout cells, except for the drug selection step.

1. After 48 h of electroporation (Subheading 3.2.2), the treated cells are subjected to drug selection (*see* **Note 24**).
2. When drug-resistant cells appear and form colonies, the cells are harvested for limiting dilution and single cell cloning (*see* **Note 25**).
3. The same procedure as **steps 3**–**7** (Subheading 3.2.3) is followed.
4. To check the gene knock-in, genetic analyses are performed using genomic DNA extracted from the divided cells in the well (*see* **Note 26**).
5. In parallel with genetic analysis (**step 4**), the cell culture is expanded (*see* **Note 22**).
6. Finally, single cell-derived clones are harvested, suspended in Cellbanker, and cryopreserved as nuclear donor cells at −80 °C or in liquid nitrogen until SCNT (*see* **Note 23**).

3.3 Somatic Cell Nuclear Transfer (SCNT)

An overview of this step is presented in Fig. 1. SCNT is the primary method for generating gene-targeted pigs. Both knockout and knock-in pig productions using SCNT follow the same process.

3.3.1 Preparation of In Vitro Matured (IVM) Oocytes

Handling of porcine oocytes is performed on a warm plate at 38.5 ° C unless otherwise indicated.

1. Porcine ovaries are collected at a local abattoir and transported to the laboratory in PBS-PVA at 30–35 °C using a thermo-container.
2. The ovaries are washed three times with CETAB solution and rinsed three times with PBS-PVA (*see* **Note 27**).
3. Cumulus-oocyte complexes (COCs) are collected by aspiration using a needle with a syringe from ovarian antral follicles with a diameter of 3.0–6.0 mm.
4. Dilute the collected COCs and follicular fluid with POE-CM, and select COCs which have at least three layers of compacted cumulus cells.
5. Wash selected COCs in three steps into NCSU23-based maturation medium, and culture them for 22 h in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 °C. Next, wash COCs with maturation medium and culture for 18–22 h without hormones in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C (*see* **Note 28**).

3.3.2 Nuclear Transfer

1. To prepare the nuclear donor, thaw the stored nuclear donor cells (Subheading 3.2.3 for knockout or 3.2.4 for knock-in), and culture in MEMα supplemented with 15% FBS to sub-confluence followed by culture in MEMα containing 0.5% FBS for starvation to synchronize the cell cycle (*see* **Note 29**).
2. IVM oocytes with expanded cumulus cells (Subheading 3.3.1) are treated with hyaluronidase and denuded of cumulus cells by gentle pipetting.
3. Denuded IVM oocytes containing the first polar body are enucleated by gentle aspiration of the polar body and adjacent cytoplasm using a beveled pipette in HEPES-TL-PVP in the presence of 0.1 μg/mL demecolcine, 5 μg/mL cytochalasin B (CB), and 10% FBS.
4. A single donor cell should be inserted into the perivitelline space of an enucleated oocyte (*see* **Note 30**).
5. The donor cell-oocyte complexes are then placed in a fusion medium and held between two electrode needles.
6. Membrane fusion should be induced with a somatic hybridizer by applying a single direct current (DC) pulse and a pre- and post-pulse alternating current (AC) field of 4 V at 1 MHz for 5 s (*see* **Note 31**).
7. The reconstructed embryos are cultured in PZM-5 (BSA) for 1–1.5 h, followed by electrical activation.
8. To induce electrical activation, the reconstructed embryos are aligned between two wire electrodes (1.0 mm apart) of a fusion chamber slide filled with activation solution. A single DC pulse of 150 V/mm should be applied for 100 μs using an electrical pulsing machine.
9. After activation, the reconstructed embryos are cultured in PZM-5 for 3 h in the presence of 5 μg/mL CB and 500 nM Scriptaid and then with 500 nM Scriptaid for another 12–14 h (*see* **Note 32**).
10. After these treatments, SCNT embryos are cultured in PZM-5 under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C until embryo transfer (*see* **Note 33**).

3.4 Cytoplasmic Injection

In the generation of knockout pigs, cytoplasmic injection is a convenient and efficient alternative to SCNT. In contrast, the efficiency of knock-in generation via cytoplasmic injection is very low in zygotes. Therefore, at present, knock-in pigs are mostly generated by SCNT using knock-in (KI) cells.

3.4.1 In Vitro Fertilization (IVF) of Porcine Oocytes

1. A straw containing frozen sperm is thawed by immersion in warm water (37 °C) for 30 s (*see* **Note 34**).

2. The sperms are then suspended in 5 mL PBS-BSA and washed and centrifuged three times at 1000 × *g* for 4 min.
3. After washing, the sperm pellets are resuspended in porcine fertilization medium (PFM) at a concentration of 1.0×10^7 cells/mL.
4. For insemination, 20 IVM oocytes (Subheading 3.3.1) are placed in a 100 μL drop of PFM containing spermatozoa ($1.0–5.0 \times 10^6$ cells/mL); the oocytes and sperms are incubated for 8 h at 38.5 °C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2 (*see* **Note 28**).
5. After insemination, the embryos are transferred into HEPES-TL-PVP; cumulus cells and excess sperms are removed by gentle pipetting.
6. Embryos showing the release of one or more polar bodies with normal cytoplasmic morphology are cultured (20–30 embryos/20–30 μL drop) in PZM-5 under paraffin oil in a plastic Petri dish maintained in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.
7. Embryos are cultured in PZM-5 (BSA) until cytoplasmic injection (*see* **Note 35**).

3.4.2 Cytoplasmic Injection to Porcine Zygotes

1. Prepare in vitro fertilized embryos (Subheading 3.4.1) in a drop of HEPES-TL-PVP supplemented with 5% FBS in a Petri dish.
2. Prepare the appropriate concentration of ZFN, TALEN-mRNA, or CRISPR-Cas9 (gRNA and Cas9-mRNA) solution for cytoplasmic injection (*see* **Note 36**).
3. The RNA solution is injected into the cytoplasm of pronuclear stage embryos using an injection pipette (*see* **Note 37**).
4. Injected embryos are washed twice with HEPES-TL-PVP.
5. Embryos are cultured (20–30 embryos/20–30 μL drop) in PZM-5 under paraffin oil in a plastic Petri dish maintained in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.
6. To generate genetically modified (gene knockout and knock-in) pigs, the injected embryos are transferred into recipient gilts (*see* **Notes 33** and **38**).

4 Notes

1. The design and construction of ZFNs are complex and difficult; therefore, we recommend their purchase from Sigma-Aldrich, if possible. The design, plasmid construction, and validation of ZFNs are performed by Sigma-Aldrich (http://

www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/custom-zfn.html).

2. We used TALENs purchased from ToolGen (http://toolgen.com); however, this service is no longer available at ToolGen. Instead, the custom TALEN service available from Thermo Fisher Scientific can be used. Alternatively, Platinum TALEN is relatively easy to construct and is capable of high-efficiency knockout in pigs as well as in other animals.
3. For DNA-based approaches in the CRISPR-Cas9 system, it is convenient to use pX330 plasmid (Addgene) containing a gRNA expression cassette and Cas9 on the same vector. To search the target site for the gene of interest, CRISPOR (http://crispor.tefor.net/) or CHOPCHOP (https://chopchop.cbu.uib.no/) tools are available for the pig genome (Sscrofa 11.1). These web tools are also useful for off-target site analysis. We typically select three or four loci in the gene of interest. Recently, custom synthesis services for crRNA and tracrRNA are useful and Cas9 protein is commercially available. The use of crRNA:tracrRNA duplex and Cas9 protein (ribonucleoprotein, RNP) is convenient and efficient for isolating gene knockout or knock-in cells (nuclear donor) and for cytoplasmic injection.
4. Plasmids as templates for in vitro transcription must be linearized using restriction enzymes because circular plasmid templates generate extremely long, heterogenous RNA transcripts. According to the manufacturer's instructions, ZFN-plasmids and TALEN-plasmids should be digested with appropriate restriction enzymes, respectively. For the CRISPR-Cas9 system, the use of custom crRNA and tracrRNA is more convenient than gRNA transcribed in vitro.
5. We adjust the concentration of each ZFN or TALEN-mRNAs to 400 ng/μL using RNase-free water. Equal amounts of the diluted RNAs are mixed to prepare ZFN- or TALEN-mRNA solution. For CRISPR-Cas9, we adjust the concentration of gRNA and Cas9-mRNA to 400 ng/μL using RNase-free water, respectively.
6. PCR-based assays are rapid and convenient for determining genome editing efficiency. To verify cleavage efficiency, we typically use cells into which genome editing molecules have been introduced by electroporation. After electroporation, the treated cells are cultured in a 6-well plate at 37 °C for 48 h, and the cleavage detection assay is then performed using the Guide-it Mutation detection Kit. Alternatively, T7 endonuclease 1 (T7E1) is available for cleaving the mismatched-heteroduplex DNA.

7. In addition to In-Fusion cloning, Gibson Assembly Cloning (New England Biolabs) is also useful for donor vector construction. A donor plasmid contains the left and right homology arms at the both ends of the desired sequence. Unless there is some particular reason, the lengths of the left and right homology arms are between 0.5 and 1.0 kb for general knock-in using genome editing tools. For drug selection to remove unmodified cells, the puromycin resistance gene (puromycin N-acetyl transferase) is usually inserted into the donor vector. Puromycin treatment in pig fibroblasts results in rapid cell death [38]. For knock-in using ssODN, which allows small gene modifications such as point mutations or small indels, using a custom synthesized ssODN service is convenient. Additionally, it is critical to either exclude the target sequences or inactivate PAM sequences in the donor template, in order to prevent Cas9 from disrupting the donor template or the edited genomic locus after it has been edited by homology-directed repair (HDR).
8. We have performed efficient knockout of various porcine genes using PFFs. We recommend using PFFs for the isolation of genetically modified cells for SCNT, although fibroblasts from neonatal or adult pigs are also available for the isolation of nuclear donor cells.
9. Generally, we isolate the primary culture of PFFs from fetuses (generally 35–40 days of gestation).
10. This step not only expands the cells but also purifies and selects for PFFs and allows for the removal of cells other than PFFs.
11. We recommend examining the karyotype (38, XY for male, 38, XX for female) of the isolated PFFs.
12. From this step, to achieve as much of an RNase-free state as possible during electroporation, DNase-/RNase-free reagents and equipment should be used for electroporation.
13. For ZFNs or TALENs, 2.5–5 μL (generally 2.5 μL) of 400 ng/μL ZFN or TALEN-mRNA solutions are added to 5×10^5 cells/50 μL of R-buffer (included with the Neon Transfection System). For CRISPR-Cas9, 5 μL of 400 ng/μL gRNA and 5 μL of 400 ng/μL Cas9-mRNA (or 2.5 μL of 3 μg/μL Cas9 nuclease protein, Takara bio) are added to 5×10^5 cells/50 μL of R-buffer. Alternatively, 5 μL of 400 ng/μL crRNA: tracrRNA duplex and 2.5 μL of 3 μg/μL Cas9 protein are added to 5×10^5 cells/50 μL of R-buffer. When using Cas9 protein instead of Cas9-mRNA, Cas9 protein and gRNA (or crRNA:tracrRNA duplex) are premixed to form a ribonucleoprotein (RNP) complex and then added to 5×10^5 cells/ 50 μL of R-buffer. Although isolation of gene knock-in cells is feasible regardless of the genome editing tool, the use of

CRISPR-Cas9 is convenient and efficient; it is also possible to establish homozygous knock-in cells using this system. As PFFs are sensitive to endotoxin, the KI donor vector plasmid should be extracted using an endotoxin-free plasmid kit. We usually use nonlinearized donor plasmid vector for knock-in, although a linearized donor vector has been used in some reports. The use of a nonlinearized donor plasmid vector can minimize unintended insertion of the donor vector into the host cell genome. For knock-in, we add 5 μL of 500 ng/μL donor plasmid vector to 5×10^5 cells/50 μL of R-buffer. When using ssODN, 1 μL of 100 μM ssODN (100 nt) is added to 5×10^5 cells/50 μL of R-buffer.

14. After adding RNAs into the cell suspension, electroporation should be started immediately to avoid RNA degradation. Optimal conditions for electroporation should be determined in advance using a reporter plasmid (e.g., EGFP-expressing plasmid, etc.). Transfection efficiency should be over 70–80%.
15. After 24 h of electroporation, we replace the complete culture medium containing 1% (v/v) antibiotic-antimycotic solution.
16. Transient cold shock treatment is effective for enhancing the cleavage activity of ZFNs or TALENs [39]. Transient cold shock treatment at 30 °C may damage electroporated cells. If this occurs, the cells can be cultured under milder conditions (32 °C for 48 or 72 h).
17. Before cell counting, pass the sample through a cell strainer to obtain a single cell suspension and eliminate aggregates for limiting dilution. We typically seed the cell suspension into five 96-well plates to obtain 96–192 single cell-derived colonies.
18. The propagation of PFFs from single cells using limiting dilution is difficult. To improve the growth performance of PFFs, adding 2-mercaptoethanol into the culture medium at a concentration of 0.1 mM is effective.
19. At 7–8 days after limiting dilution, small colonies can be observed (Fig. 3). Wells containing more than two colonies of cells are excluded from further experiments. Further, wells containing extremely large colonies compared with other colonies are also excluded from further experiments because such colonies are likely not derived from single cells.
20. After stopping the trypsin reaction by adding culture medium directly to the 96-well plates, the cell suspension is mixed well by gentle pipetting. Subsequently, half of the cells are passaged into 48-well plates, and the remaining cells are allowed to adhere to the plate (generally for 2–3 h) for mutation analysis using direct PCR. When using CRISPR-Cas9, we typically

analyze and passage 48–96 single cell-derived colonies for the mutation analysis.

21. After aspirating the medium, PCR mixtures of MightyAmp DNA polymerase ver.2 (Takara Bio) are directly added to the well in which half of the cells are adherent as described in **step 7** (Subheading 3.2.2). Next, the cells in the well are scratched using a pipet tip, and the liquid PCR sample is transferred to 96-well PCR plates for mutation analysis. Generally, small insertion and deletion (indel) mutations can be identified by direct sequencing in most cell clones analyzed. Subcloning of the PCR products using the TOPO Cloning Kit (Thermo Fisher Scientific) and sequencing may be required to identify the mutations because of the presence of complicated indel mutations or sequences lacking sequencing primer binding sites as a result of large deletions and insertions. It is important to quickly identify the mutation because culturing many single cell-derived clones continues simultaneously.
22. After passage to a 48-well plate, the cells can typically be passaged every 2–3 days (48-well → 24-well → 12-well → 6-well plate → 60 mm dish → 100 mm dish). The time interval of passage can be used as an indicator to obtain good candidates for nuclear donor cells with potential proliferation abilities.
23. At this step, the passage number of the cell clones will reach approximately P10. Approximately 30–40 days are required to isolate nuclear donor cells (from electroporation to cryopreservation of nuclear donor cells). We thus recommend repeating the genetic analysis of the cryopreserved cells to be used as nuclear donors at this step.
24. We usually use puromycin (2.0–2.5 μg/mL) for drug selection. Puromycin treatment in pig fibroblasts results in rapid cell death compared with treatment of other drugs such as G418 or hygromycin B [38]. The use of ssODN is suitable for inducing point mutations or small indels at the locus of interest. However, no drug selection step is available for ssODN-mediated knock-in, because ssODN (generally 100–200 nt) have no drug-resistant genes. We thus recommend the use of CRISPR-Cas9 for isolating gene knock-in cells.
25. Depending on the gene editing efficiency, drug-resistant cell colonies appear after 3–5 days of drug selection. However, colonization may not be observed if many drug-resistant cells exist. We thus perform limiting dilution to obtain single cell-derived cell clones with high proliferative performance; however, colonies can also be picked using cloning cylinders. After limiting dilution, cell culture is performed in normal culture medium without any drugs.

26. We usually perform 5′- and 3′-junction PCR between the host genome and donor sequences using knock-in allele-specific primers. In some cases, nested PCR is also performed. Additionally, we perform PCR analysis and DNA sequencing to determine the accuracy of the knock-in and the state (homozygous or heterozygous).
27. Until the aspiration of follicles, keep the washed ovaries in PBS-PVA in a water bath at 38.5 °C.
28. For preparation of in vitro fertilized embryos (Subheading 3.4.1), wash selected COCs in three steps into POM-based maturation medium, and culture them for 20 h, followed by culture for 24 h without dbcAMP and hormones, in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.
29. Synchronization of nuclear donor cells at the G0/G1 stage is a key factor for the success for SCNT. For further technical details on SCNT, refer to the excellent separate book in this series [37].
30. Relatively small-size cells are suitable as nuclear donors because it is assumed that G0/G1 stage cells are smaller than those in the G2/M stage.
31. Determination of an optimal a single direct current (DC) pulse should be tested depending on the instrument used. We typically induce membrane fusion by applying a single direct current (DC) pulse (267 V/mm, 20 μs) using a somatic hybridizer (LF201, Nepa Gene).
32. Treatment with histone deacetylase inhibitor, Scriptaid, is effective for improving the in vitro development of SCNT embryos. The morphology of cloned blastocysts treated with Scriptaid is better than that of untreated blastocysts, and the number of constituent cells is high [40].
33. We choose the sites (oviducts or uterine horn) of embryo transfer depending on the stage of the embryos. Embryos cultured for 1–2 days are surgically transferred into the oviducts of recipients, approximately 10–13 h after the estimated time of ovulation. Embryos cultured for 5–6 days are transferred at the blastocyst stage to the uterine horns of recipients, approximately 105–107 h after the estimated time of ovulation. The genotypes of all cloned offspring from SCNT embryos should be genetically identical to those of the nuclear donor cells.
34. We use frozen epididymal sperms from a boar at a concentration of 1.0×10^9 cells/mL as described by Kikuchi et al. [41] prior for use in IVF.
35. We typically use the in vitro fertilized embryos for cytoplasmic injection 8.5–9.0 h after insemination.

36. Generally, we use 2–10 ng/μL of ZFN or TALEN-mRNAs diluted with RNase-free water. For the CRISPR/Cas9 system, 2–10 ng/μL of gRNA and 10 ng/μL Cas9-mRNA or 10–20 ng/μL Cas9 proteins are used for injection. When using Cas9 protein, Cas9 protein is premixed with gRNA (generally for 5–10 min at 20–25 °C) to form a RNP complex just before injection. The use of the crRNA:tracrRNA duplex is also feasible instead of gRNA transcribed in vitro.
37. We typically inject approximately 10 pL of solution into the cytoplasm of the in vitro fertilized embryos.
38. Unlike cloned offspring from SCNT embryos, in some of offspring obtained from embryos by cytoplasmic injection, more than three different allele variants can be found in single individuals because of mosaicism. Therefore, the phenotypes of these mosaic founders should be interpreted with care, particularly for human disease models. Mosaicism in cytoplasmic injection is particularly troublesome for large animals as described in the introduction section. To date, there are no clear strategies for reducing mosaicism. To reduce mosaicism using the CRISPR-Cas9 system, Cas9 protein rather than RNA may be useful [42].

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Chapter 22

GEEP Method: An Optimized Electroporation-Mediated Gene Editing Approach for Establishment of Knockout Pig Lines

Fuminori Tanihara, Maki Hirata, and Takeshige Otoi

Abstract

Pigs are excellent large animal models owing to their several physiological and anatomical similarities to humans. Somatic cell nuclear transfer using gene-modified cells is the mainstream approach for generating genetically modified pigs. Recent advances in improving gene editors such as the CRISPR/Cas9 system have enabled direct gene modification in zygotes/embryos. Here, we describe the gene editing by electroporation of Cas9 protein (GEEP) method, an optimized electroporation-mediated method for the introduction of CRISPR/Cas9 into porcine zygotes/embryos. The simplicity and micromanipulation-free procedures are the major advantages of this method.

Key words CRISPR/Cas9, Electroporation, In vitro fertilization, Pig, Zygote

1 Introduction

Pigs are excellent large animal models in biomedical research because of their similarity to humans in several aspects, particularly in terms of anatomy and physiology [1–3]. Electroporation-mediated gene editing is a micromanipulation-free method in which large numbers of gene-edited zygotes/embryos can be prepared by introducing gene editors directly into the zygotes, and it has become a widely used gene editing method in mice [4]. We previously developed the gene editing by electroporation of Cas9 protein (GEEP) method [5] in which the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system [Cas9 protein and guide RNA (gRNA)] are introduced into porcine in vitro-fertilized zygotes by electroporation to disrupt a target gene (Fig. 1).

Since porcine in vitro-developed zygotes are more sensitive to electronic stimulation than those of mice, the electroporation conditions established for mice, such as the voltage, pulse number, and pulse duration, should be reduced when applying the method to

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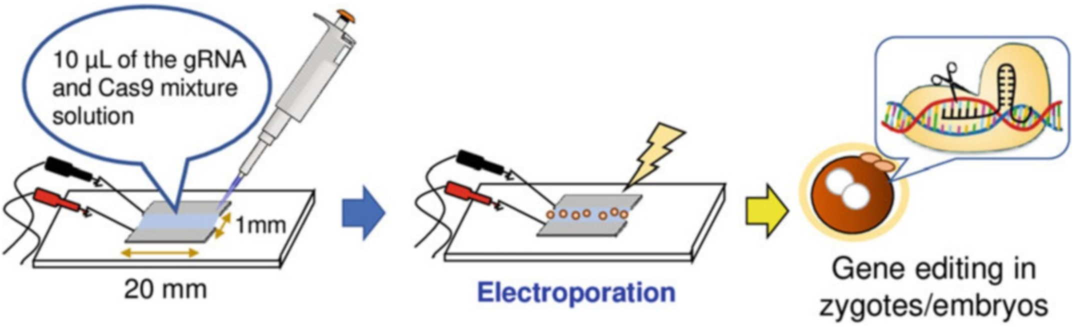

10 µL of the gRNA and Cas9 mixture solution
1mm
20 mm
Electroporation
Gene editing in zygotes/embryos

Fig. 1 Schematic of the electroporation of zygotes using platinum electrodes. Schematic of the electroporation chamber with platinum electrodes (BEX Co. Ltd.). In the gap between the electrodes, porcine zygotes are placed with the gRNA/Cas9 solution and electroporation is then performed

pigs [5, 6]. However, this requirement imposes a limit on the weight of molecules to be introduced using electroporation; thus, delivery of the CRISPR/Cas9 system is recommended for ribonucleoproteins and not mRNA. We previously reported an improvement for the applicability of the GEEP method in which an increase of the Cas9 concentration improved the mutation efficiency [7, 8] as one of the countermeasures against mosaicism that is often observed in zygotic gene editing using CRISPR/Cas9 [9]. Moreover, we demonstrated that a one-step gene modification strategy targeting multiple genes is also feasible with the GEEP method [8]. Here, we describe the detailed conditions and optimized procedures for electroporation-mediated gene editing in porcine zygotes.

2 Materials

Where water is required, use ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C). Prepare all solutions using cell culture-grade reagents.

2.1 Common Materials for Each Step

1. Heating plate: used for handling of oocytes/zygotes/embryos outside the incubator.
2. CO_2/multigas incubator: used for in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) of oocytes/zygotes/embryos.
3. Glass pipette: used for mouth-operated treatment of oocytes/zygotes/embryos.
4. Stereomicroscope.
5. Mineral oil or liquid paraffin: used for covering media.

6. 4-well dishes: used for culturing of oocytes/zygotes/embryos.
7. Sterilized 15 mL tube.
8. 0.22 μm syringe filter: used for filter sterilization.

2.2 Oocyte Collection

1. Physiological saline for washing the ovaries: weigh 9.0 g of NaCl and transfer to a 1000 mL graduated cylinder. Fill up to 1 L with water. After autoclave sterilization, add 100 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate.
2. Oocyte washing/collecting medium: use modified phosphate-buffered saline (Embryotech, Nihon Zenyaku Co, Fukushima, Japan) supplemented with 50 μg/mL gentamicin. If unavailable, use tissue culture medium 199 with Hank's salts supplemented with 5% (v/v) fetal bovine serum, 20 mM HEPES, 100 IU/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate.
3. Sterilized plastic petri dish: prepare 35 mm, 60 mm, and 100 mm-diameter dishes.
4. Constant temperature water bath.
5. Surgical blade.

2.3 *In Vitro* Production of Porcine Oocytes/Embryos

1. IVM 2 medium: tissue culture medium 199 with Earle's salts supplemented with 10% (v/v) porcine follicular fluid (*see* **Note 1**), 0.6 mM cysteine, 50 μM β-mercaptoethanol, 50 μM sodium pyruvate, 2 mg/mL D-sorbitol, and 50 μg/mL gentamicin. After filtering through a 0.22 μm syringe filter, store the medium at 4 °C.
2. IVM 1 medium: add 10 IU/mL equine chorionic gonadotropin (eCG) and 10 IU/mL human chorionic gonadotropin (hCG) to IVM 2 medium. After filtering through a 0.22 μm syringe filter, store the medium at 4 °C.
3. IVF medium for sperm washing and coculture of oocytes with sperm: use porcine fertilization medium (PFM, Research Institute for the Functional Peptides Co., Yamagata, Japan) or PGMtac4 prepared according to the protocol described by Yoshioka et al. [10]. In brief, add 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 1 mM glucose, 0.2 mM Na-pyruvate, 4 mM $Ca\text{-(lactate)}_2 \cdot 5H_2O$, 2.5 mM theophylline, 1 μM adenosine, 0.25 μM L-cysteine, 10 μg/mL gentamicin, and 3 mg/mL polyvinyl alcohol to water. After adjusting to pH 7.3 and filtering through a 0.22 μm syringe filter, store the medium at 4 °C.

4. IVC medium for embryo culture: use porcine zygote medium (PZM-5) purchased from the Research Institute for the Functional Peptides Co., or prepare according to the protocol described by Yoshioka et al. [10]. In brief, add 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4$•$7H_2O$, 25 mM $NaHCO_3$, 0.20 mM Na-pyruvate, 2 mM Ca-$(lactate)_2$•$5H_2O$, 2 mM L-glutamine, 5 mM hypotaurine, 20 mL/l BME amino acids, 10 mL/l MEM nonessential amino acids, 10 μg/mL gentamicin, and 3 mg/mL polyvinyl alcohol to water. After adjusting to pH 7.3 and filtering through a 0.22 μm syringe filter, store the medium at 4 °C.
5. Cell counter: used for calculating sperm concentration.

2.4 Electroporation-Mediated Gene Editing

Alt-R CRISPR crRNAs and the tracrRNA system, supplied by the Integrated DNA Technologies (IDT; Coralville, IA, USA), are used for preparing the gRNA.

1. Nuclease-Free Duplex Buffer (IDT): divide into aliquots and store at −20 °C or −80 °C.
2. 1 μg/μL tracrRNA, 1 μg/μL crRNA: dilute with Nuclease-Free Duplex Buffer and store at −20 °C or −80 °C. Up to approximately five freeze-thaw cycles will have no effect on the gene editing efficiency.
3. Cas9 protein (3 μg/μL; Guide-it™ Recombinant Cas9; Takara Bio, Inc., Shiga, Japan): divide into aliquots and store at −80 °C.
4. Opti-MEM™ I Reduced Serum Medium (Gibco/Invitrogen Co.): used for washing zygotes directly before electroporation.
5. Block incubator: capable of maintaining temperatures above 98 °C.
6. Platinum chamber slide for electroporation (LF501PT1-20; BEX, Tokyo, Japan).
7. CUY21EDIT II electroporator (BEX).
8. 500 mM NaOH.
9. 1 M Tris–HCl (pH 8.0): 1 mol/l tris(hydroxymethyl)-aminomethane with pH adjusted by hydrochloric acid.

3 Methods

All procedures involving the treatment of porcine oocytes/zygotes/embryos should be carried out at 37–38 °C on a heating plate unless otherwise specified. Except during manipulation, oocytes/embryos are cultured at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

3.1 Oocyte Collection and IVM

1. Obtain pig ovaries from prepubertal gilts (around 6 months old) from a local slaughterhouse, which are then maintained in physiological saline at 30 °C until use.
2. Wash the ovaries three times with 37.5 °C physiological saline solution containing antibiotics. The ovaries should be maintained at 37.5 °C using a constant temperature water bath until the follicles are cut.
3. Slice 3–6 mm-diameter follicles on the ovarian surface on a sterilized dish filled with oocyte washing/collecting medium using a surgical blade.
4. Collect the contents of the follicles and oocyte washing/collecting medium in a sterilized 15 mL tube and place in a water bath at 37.5 °C for at least 15 min.
5. Remove the supernatant and resuspend the deposited cells/cumulus-oocyte complexes (COCs) in oocyte washing/collecting medium. Transfer this solution into a sterilized petri dish (60 mm or 100 mm).
6. Collect the visualized COCs using a mouth-operated glass pipette under a stereomicroscope (*see* **Note 2**). Use a pipette with a large inner diameter and be careful not to remove the cumulus cells attached to the surface of oocytes.
7. Wash COCs in a 35 mm petri dish with 2 mL of IVM 1 medium covered with mineral oil.
8. Transfer and wash COCs in a 35 mm petri dish with 2 mL of fresh IVM 1 medium. Repeat this step twice.
9. Culture approximately 50 COCs in 500 μL of IVM 1 medium, covered with mineral oil, for 22 h in 4-well dishes.
10. Transfer to and wash COCs in a 35 mm petri dish with 2 mL of IVM 2 medium without eCG and hCG. Repeat two times.
11. Transfer the 50 COCs into a 4-well dish with 500 μL of IVM 2 medium without eCG and hCG, and culture for an additional 22 h.

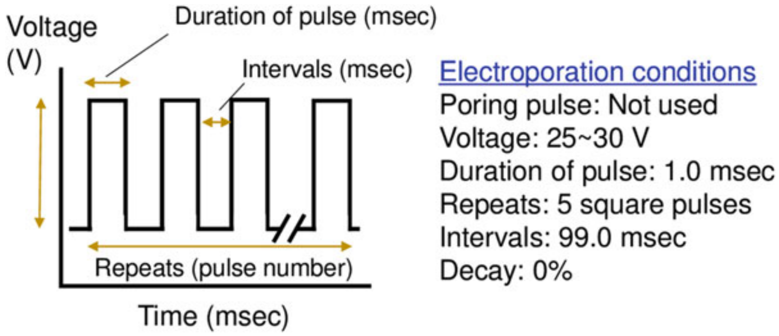
3.2 IVF

1. After IVM culture for 44 h, subject the matured COCs to IVF. Transfer and wash COCs in a 35 mm petri dish with 2 mL of PFM or PGMtac4. Repeat two times.
2. Transfer freeze-thawed ejaculated spermatozoa into 5 mL of PFM or PGMtac4, and wash by centrifugation at 500 × *g* for 5 min at room temperature (25–27 °C).
3. Remove the supernatant, resuspend the pelleted spermatozoa in 300–500 μL of PFM or PGMtac4, calculate sperm concentration using cell counter, and adjust to the optimized sperm concentration (*see* **Note 3**).

4. Load the sperm-containing PFM/PGMtac4 in 4-well dishes (500 μL/well) and cover with mineral oil.
5. Transfer approximately 50 COCs/well (500 μL) into sperm-containing PFM/PGMtac4 and co-incubate for 3–10 h (*see* **Note 4**).
6. After co-incubation, transfer the putative zygotes into a 35 mm petri dish with 2 mL of PFM or PGMtac4, and denude the cumulus cells and the attached spermatozoa by mechanical pipetting using a pipette that is slightly larger than the diameter of the oocytes.
7. Transfer and wash the putative zygotes with 500 μL drops of PZM-5 covered with mineral oil prepared in a 35 mm petri dish.
8. Transfer 50 putative zygotes into a 4-well dish with 500 μL of PZM-5 and culture until electroporation. Generally, electroporation is performed 10–12 h after the start of IVF.

3.3 Electroporation-Mediated Delivery of the CRISPR/Cas9 System into Zygotes and IVC

1. Prepare the gRNA and Cas9 mixture solution. Mix 7.6 μL of duplex buffer with 1 μL tracrRNA stock solution and 1 μL crRNA stock solution. After incubation for 3 min at 95 °C, add 0.34 μL Cas9 protein solution (final concentration: 100 ng/μL gRNA and 100 ng/μL Cas9 protein).
2. Wash at least 50 fertilized zygotes with a 100 μL drop of Opti-MEM™ I Reduced Serum Medium without covering in mineral oil.
3. Place the zygotes in line in the electrode gap on a chamber slide (LF501PT1-20) filled with 10 μL of the gRNA and Cas9 mixture solution (*see* **Note 5**).
4. Electroporate the zygotes (five 1 ms pulses at 25 V, without poring pulse) using a CUY21EDIT II electroporator (Fig. 2).



Electroporation conditions
Poring pulse: Not used
Voltage: 25~30 V
Duration of pulse: 1.0 msec
Repeats: 5 square pulses
Intervals: 99.0 msec
Decay: 0%

Fig. 2 Detailed conditions of electroporation for porcine zygotes. Schematic of the electroporation conditions and detailed parameters of our current method. Normally, five repeats of a square 1 ms pulse of 25–30 V with 99 ms intervals are used. Poring pulse will affect viability in porcine zygotes

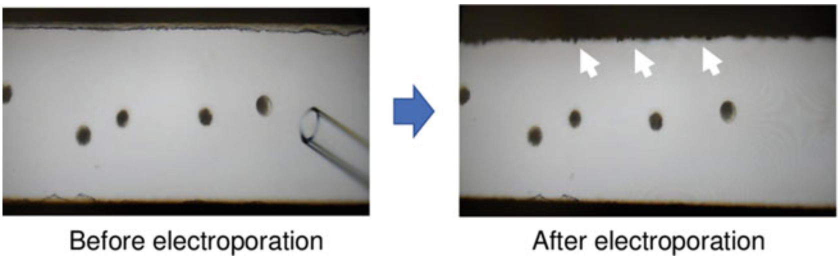


Fig. 3 Bubbles on the platinum electrode surface after electroporation. After electroporation, bubbles are observed on the platinum electrode surface (arrow). The formation of bubbles is an indicator of current flow

The formation of bubbles on the platinum electrode surface is an indicator of the current flow (Fig. 3).

5. Wash the electroporated zygotes with a 500 μL drop of PZM-5 covered with mineral oil prepared in a 35 mm petri dish.
6. Transfer 50 putative zygotes into a 4-well dish with 500 μL of PZM-5.
7. Culture the zygotes until embryo transfer or development to the blastocyst stage for 7 days in PZM-5.
8. Collect blastocysts individually in 10 μL water, add 1 μL of 500 mM NaOH, and incubate for 10 min at 98 °C. After incubation, add 1 μL of 1 M Tris–HCl, and use as the polymerase chain reaction template to evaluate the gene editing event using specific primers targeting the genomic regions flanking the gRNA target sequences (*see* **Note 6**).

4 Notes

1. Follicular fluid is prepared by aspirating from follicles on the surface of the ovaries (generally obtained from a local slaughterhouse) using an 18G needle connected to a sterilized syringe. After centrifugation at 1500 × *g* for 60 min at 4 °C, collect the supernatant and store at −20 °C. The quality of the follicular fluid from different sources (ovaries) will be uneven; thus, multiple samples should be mixed, centrifuged again, and stored at −20 °C until use.
2. Collect COCs that are surrounded by at least three layers of cumulus cells with a uniform cytoplasm.
3. IVF conditions for successful penetration using frozen/thawed sperm may differ among samples from different sources (boars). Preliminarily evaluate the optimal sperm concentration according to the monospermic penetration rate and blastocyst formation rate as indicators. To evaluate detailed fertilization statuses (inseminated sperm and pronucleus), performing

aceto-orcein staining (1% orcein in 45% acetic acid) after fixation with acetic acid:ethanol (1:3 v/v) for 48–72 h is recommended [11], as aceto-ethanol solution removes the high amount of cytoplasmic lipid droplets in porcine zygotes.

4. Preliminarily evaluate the optimal duration of sperm-oocyte co-incubation according to the monospermic penetration rate and blastocyst formation rate as indicators.
5. Adjust the volume of the gRNA and Cas9 mixture solution depending on the length of the electrode; the required volume of solution is half if the length of electrode is half.
6. Gene editing efficiency depends on the gRNA design. If the number of resulting gene-edited blastocysts is low, the gRNA should be redesigned.

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Chapter 23

Genome Editing Mediated by Primordial Germ Cell in Chicken

Jae Yong Han and Hong Jo Lee

Abstract

Genome editing technology has facilitated the studies on exploring specific gene functions in diverse living organisms. The technology has also contributed to creating high-value livestock in industry fields in terms of enhancing productivity or acquiring disease resistance. Particularly, applying genome editing technologies in avian species has been emphasized in both academic and industrial fields due to their unique developmental patterns as well as application possibilities. To accomplish genome editing in avian species, gene integration into chicken primordial germ cell (PGC) genome using a virus or transposition systems has been widely used, and recently developed programmable genome editing technologies including clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas9) systems enable to edit the genetic information precisely for maximizing the application potentials of avian species. In these regards, this chapter will cover the methods for producing genome-edited chickens, particularly by CRISPR/Cas9 technologies allowing targeted gene insertion, gene knockout, and gene tagging.

Key words Genome editing, Chicken, Primordial germ cell, CRISPR/Cas9

1 Introduction

Genome editing in livestock provides enormous benefits to industry fields as well as research fields. Regulation of specific gene expression that is related to growth rate and virus susceptibility could contribute to enhancing productivity and acquiring viral disease resistance in livestock [1]. There has also been a large demand for genome editing in avian species because the avian species have been recognized as important model animals due to their unique developmental patterns that differ from mammals; moreover, they are one of the most important protein resources for human society [2].

Genome editing technology in avian species has been largely focused on chickens. After the production of the first transgenic chicken-mediated virus vector system, diverse approaches to producing genome-edited chicken have been tried [3–12]. Especially,

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primordial germ cell (PGC)-mediated germline transmission system in avian species has been considered the most efficient method for transmitting genetic information to the next generation [13–15], and this germline transmission system was adapted to diverse genome editing technologies including *piggyBac* transposition for producing the transgenic chickens [16–19].

Particularly, applying a recently developed programmable genome editing technology, CRISPR/Cas9 [20], to the chicken PGC-mediated germline transmission system has facilitated the development of novel chicken lines that are valuable in the poultry industry as well as a research area. It enabled us to produce diverse avian models for chicken sex determination [21] and germ cell tracing [22] and to establish the genome-edited chickens having mutations on genes that relate to economic traits [23]. Moreover, the technology has been expanded to develop disease-resistant chicken lines [24, 25] and to restore avian germplasm [26] indicating the versatility of the CRISPR technologies in avian biotechnology.

In these regards, the book chapter is going to describe methods to produce genome-edited chickens especially mediated by CRISPR/Cas9 technologies including base editing, from in vitro culture of PGCs and its genome modification.

2 Materials

2.1 Isolation and In Vitro Culture of PGCs

1. Chicken embryos at Hamburger and Hamilton (HH) stages 26–28 [27].
2. Sharpened forceps.
3. Trypsin/ethylenediaminetetraacetic acid.
4. PGC culture medium: Knockout Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) fetal bovine serum (FBS), 2% (vol/vol) chicken serum, 1× nucleosides, 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, and 1× antibiotic-antimycotic, and 10 ng/mL human basic fibroblast growth factor (bFGF).
5. 1× Hank's balanced salt solution (HBSS) without $CaCl_2$ or $MgCl_2$.
6. Mouse embryonic fibroblast (MEF, ICR strain mouse) (*see* **Note 1**).
7. Mitomycin C.
8. 0.1% gelatin.

2.2 PGC Transfection and Selection

1. Lipofectamine 2000 (Thermo Fisher—Invitrogen).
2. Opti-MEM medium.

3. PBS, without Ca^{2+} or Mg^{2+}.
4. HBSS without Ca^{2+} or Mg^{2+}.
5. pSpCas9(BB)-2A-Puro (PX459) V2.0 from Feng Zhang (Addgene plasmid # 62988) [28] and donor plasmid containing reporter genes and guide RNA (gRNA) targeting sites, concentrated at 1 μg/μL.
6. pcDNA3.1-pCMV-nCas-PmCDA1-ugi pH 1-gRNA (HPRT) plasmid (Target-AID) from Akihiko Kondo (Addgene #79620) [29], concentrated at 1 μg/μL.
7. Hemocytometer.
8. Fluorescent microscope.
9. Geneticin Selective Antibiotic (G418).

2.3 Validation of Genome-Edited PGC

1. Genomic DNA extraction kit.
2. Fluorescent microscope.
3. PCR product clean-up kit.
4. pGEM-T easy vector (Promega).
5. ABI 3739XL DNA Analyzer (Applied Biosystems).
6. PCR instrument.
7. PCR reagents: PCR buffer, dNTP, and Taq polymerase.
8. Agarose.
9. 50× TAE buffer: 242 g of Tris-base, 57.1 mL of acetate, and 18.61 g of disodium EDTA in 1 L of distilled water.
10. Electrophoresis equipment.
11. T7E1 endonuclease.

2.4 PGC Transplantation

1. Small-diameter (25 μm) glass micropipette (made with microelectrode pipette puller Shutter Instrument Co.) and a micro grinder (Narishige).
2. Syringe-controlled pipette.
3. HBSS without $CaCl_2$ or $MgCl_2$.
4. Recipient chicken embryos at HH stages 14–17.
5. PBS, without Ca^{2+} or Mg^{2+}.
6. Pipette washing solution. 0.1% hydrogen peroxide in autoclaved distilled water.
7. Forceps.
8. Stereomicroscope with illuminator.
9. Parafilm.
10. Donor PGCs prepared at 3×10^3 cells/μL in HBSS.

2.5 Testcross for Germline Transmission

1. Wild-type (WT) hens.
2. 1 mL syringe.
3. PBS, without Ca^{2+} or Mg^{2+}.

2.6 Validation of Genome-Edited Chicken

1. Genomic DNA extraction kit.
2. PCR instrument.
3. PCR reagents: PCR buffer, dNTP, and Taq polymerase.
4. Agarose.
5. 50× TAE buffer: 242 g of Tris-base, 57.1 mL of acetate, and 18.61 g of disodium EDTA in 1 L of distilled water.
6. Electrophoresis equipment.
7. Fluorescent excitation lamp with detection filters (BLS Ltd.).
8. PCR product clean-up kit.

3 Methods

Here, we described the methods to produce genome-edited chickens mediated by CRISPR/Cas9 technology, particularly the CRISPR/Cas9 and nonhomologous end joining (NHEJ) pathway (CRISPR/Cas9-NHEJ)-mediated genome editing (*see* Fig. 1), allowing targeted gene insertion [30], gene knockout [21], and gene tagging [22] (*see* Fig. 2a) as well as base editing [23].

3.1 Isolation and In Vitro Culture of PGCs

1. Incubate fresh chicken eggs at Eyal-Giladi and Kochav (EGK) stage X [31] at 37 °C for 5.5 days (HH stages 26–28) for gonadal PGC isolation.
2. Extract embryonic gonads from embryos at HH stages 26–28 with sharpened forceps, and incubate with 500 μL of 0.05% trypsin/EDTA at 37 °C incubator for 5 min.
3. Add 50 μL FBS for inactivation, and centrifuge (200 *g*, 5 min) and remove the supernatant.
4. Resuspend the dissociated gonadal cells and maintained them with PGC media.
5. The cultured PGCs are subcultured onto mitomycin C-inactivated MEFs in 5–6-day intervals by gentle pipetting without any enzyme treatment.

3.2 PGC Transfection and Selection

1. For targeted gene insertion in chicken PGCs, mix 2 μg green fluorescent protein (GFP) and gRNA targeting sites containing donor plasmid and 2 μg CRISPR/Cas9 plasmid with 500 μL Opti-MEM, and incubate for 5 min at room temperature (RT). To induce targeted nucleotide substitution, mix 3 μg of

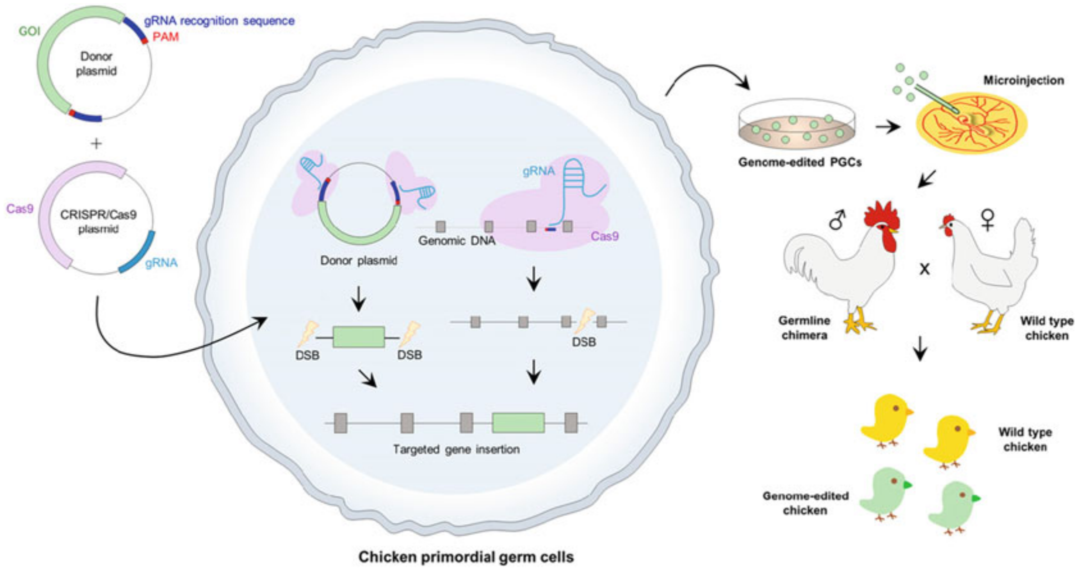

Fig. 1 Schematic representation of CRISPR/Cas9-NHEJ-mediated genome editing method. The introduction of the CRISPR/Cas9 plasmid encoding Cas9 protein and gRNA, along with the donor plasmid containing gRNA recognition sequences and the gene of interest, results in DNA DSBs in both chicken genomic DNA and the introduced donor plasmid. Consequently, targeted gene insertion is occurred in the chicken PGCs. Genome-modified PGCs were established in vitro using drug selection, and genome-modified PGCs were transplanted into recipient embryos to produce genome-modified chickens. (Figures from Lee et al. [30])

base editing plasmid containing gRNA with 500 μL Opti-MEM and incubate for 5 min at RT.

2. Mix Lipofectamine 2000 reagent with 500 μL Opti-MEM and incubate for 5 min at RT.
3. Mix the plasmids with Opti-MEM and Lipofectamine 2000 reagent with Opti-MEM and incubate for 20 min at RT.
4. During incubation, harvest cultured PGCs, and centrifuge (200 *g*, 5 min). Discard the supernatants.
5. Add 1 mL HBSS to the harvested PGCs and gently pipette.
6. Determine the number of PGCs using a hemocytometer and seed 5×10^5 PGCs in a 12-well plate with 1 mL DNA-Lipofectamine complex and incubate for 4 h at 37 °C in a CO_2 incubator.
7. Harvest the transfected PGCs and centrifuge (200 *g*, 5 min). Remove the supernatants.
8. Wash the PGCs with 1 mL PBS three times and suspend them with a PGC culture medium with antibiotics.
9. One or 2 days after transfection, add 300 μg/mL G418 to the culture media for selection (*see* **Note 2**).

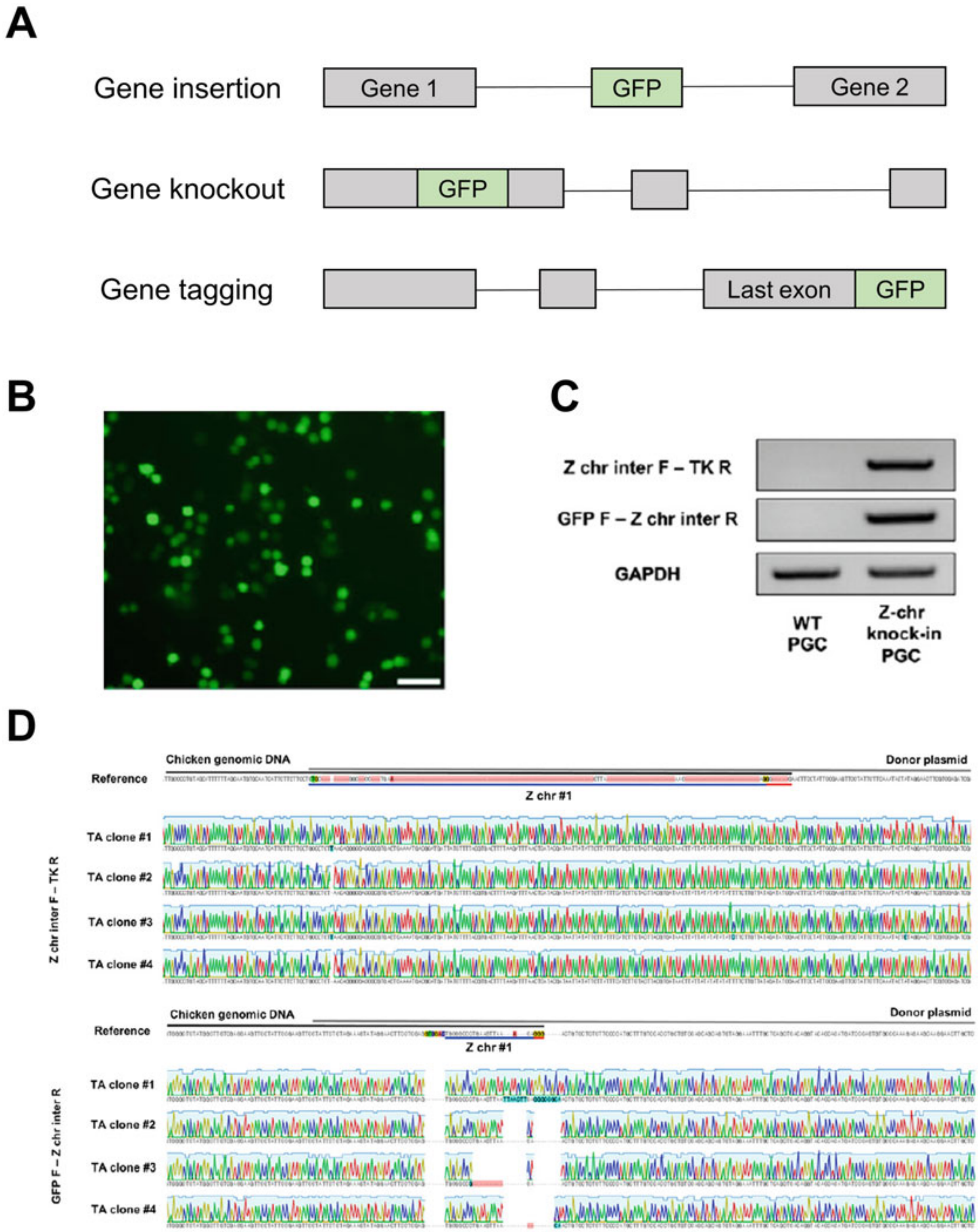

Fig. 2 Targeted gene insertion in chicken PGCs. (**a**) Schematic representation of targeted gene insertion, gene knockout, and gene tagging by CRISPR/Cas9-NHEJ method. (**b**) Expression of GFP in chicken primordial germ cells (PGCs). Scale bars, 100 μm. (**c**, **d**) Genomic DNA analysis of targeted gene insertion in chicken PGCs by knock-in-specific PCR and sequencing of the TA-cloned PCR product. Blue bars indicate gRNA recognition sequences and red bars indicate PAM sequences. Nucleotide sequences of chicken genomic DNA and the donor plasmid are shown. (Figures from Lee et al. [30])

3.3 Validation of Genome-Edited PGC

1. After drug selection, monitor reporter gene expression of PGCs using a fluorescent microscope, and analyze reporter gene expression by flow cytometry if donor plasmids contain reporter gene expression cassette (*see* Fig. 2b).
2. Extract genomic DNA of the transfected PGCs using a genomic DNA extraction kit according to the manufacturer's protocol.
3. Perform PCR of the genomic DNA using knock-in-specific primers and identify genome-edited allele-specific bands by electrophoresis (*see* Fig. 2c).
4. PCR amplicons were cloned into a pGEM-T easy vector and sequenced with an ABI 3730XL DNA Analyzer (*see* Fig. 2d).
5. Perform the T7E1 assay to identify nucleotide substitution in base-edited PGCs.
6. Amplify the genomic region encompassing the base editing vector targeting sites with specific primers by PCR.
7. Reanneal the PCR amplicons to form heteroduplex DNA structures.
8. Treat T7E1 endonuclease to reannealed heteroduplex amplicons at 37 °C for 15 min and identify cleaved amplicons by electrophoresis (*see* **Note 3**, Fig. 3a).
9. To confirm mutations on target sites, the PCR amplicons were cloned into a pGEM-T easy vector and sequenced with an ABI 3730XL DNA Analyzer (*see* Fig. 3b).

3.4 PGC Transplantation

1. Incubate recipient eggs up to HH stages 14–17 at 37 °C in the air with 60–70% relative humidity.
2. Make a small window on the pointed end of the recipient egg, and a 2 μL aliquot containing more than 3000 PGCs was microinjected with a micropipette into the dorsal aorta of the recipient embryo.
3. Seal the egg window of the recipient embryo with paraffin film, and the egg was incubated with the pointed end down until further screening and hatching at 37 °C in the air with 60–70% relative humidity.

3.5 Testcross for Germline Transmission

1. After the sexual maturation of recipients, collect the semen from recipient roosters twice in 1 week.
2. Introduce 50 μL semen from mature male recipients to WT laying hens.
3. Collect eggs from WT laying hens the day after artificial insemination, and incubate the eggs with the pointed end down until hatching at 37 °C in the air with 60–70% relative humidity.

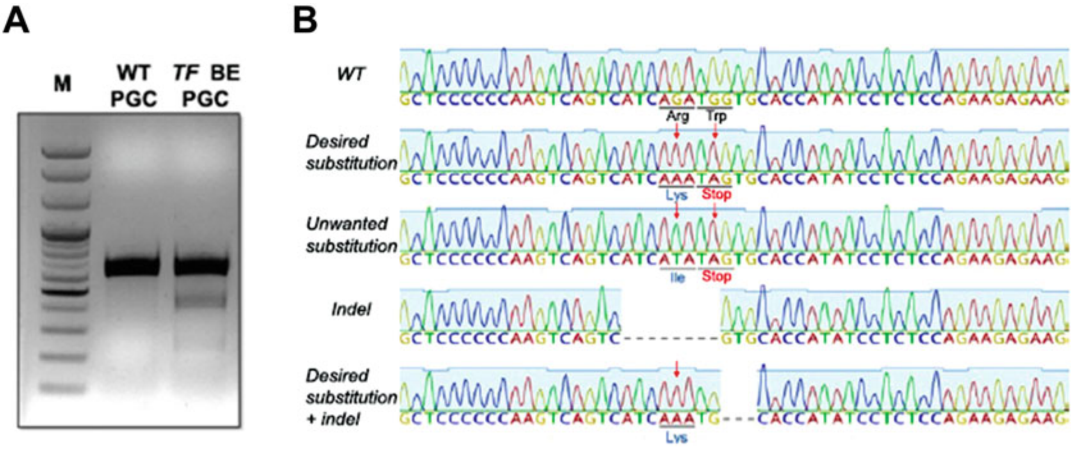

Fig. 3 Base editing in chicken PGCs. (**a**, **b**) T7E1 assay, representative Sanger sequencing chromatograms, and sequencing analysis patterns of PGCs transfected with the base editing plasmid (*TF* BE PGC). Wild-type (WT) PGCs were used as controls. (Figures from Lee et al. [23])

4. Donor PGC-derived progenies can be distinguished from recipient endogenous PGC-derived progenies by feather color (*see* Fig. 4a, **Note 4**).

3.6 Validation of Genome-Edited Chickens

3.6.1 Validation of Targeted Gene Insertion and Base Editing

1. To validate genome-edited chickens that express fluorescent reporter protein, donor PGC-derived chickens are monitored using a fluorescent microscope or excitation lamp with detection filters (*see* Fig. 4b). In targeted gene tagging, reporter gene expression can be detected in specific cell types depending on the target genes (*see* Fig. 4c).
2. To confirm the targeted gene insertion of transgenic chickens, perform PCR of the chicken's genomic DNA using knock-in-specific primers, and identify the knock-in-specific band by electrophoresis. Then the PCR products were purified with a PCR product clean-up kit and then cloned directly into the pGEM-T easy vector. The cloned plasmids were sequenced using an ABI Prism 3730 XL DNA Analyzer.
3. To validate base editing in chicken, extract genomic DNA and perform PCR on the targeted regions, and the PCR amplicons were sequenced as described previously.
4. Potential off-target effects of CRISPR/Cas9 are predicted using the web-based software programs, CRISPR Design [32, 33] (*see* **Note 5**).

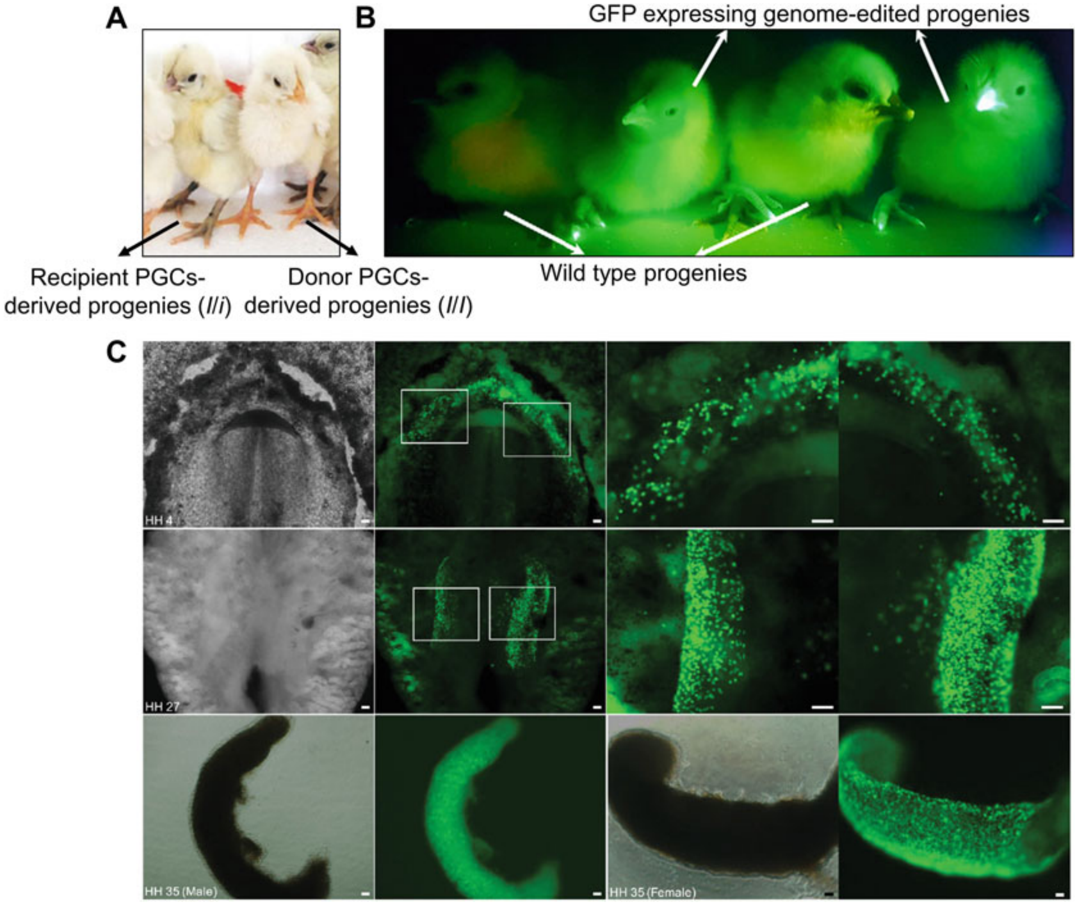
A
B
GFP expressing genome-edited progenies
Wild type progenies
Recipient PGCs-derived progenies (*I/i*)
Donor PGCs-derived progenies (*I/I*)
C
HH 4
HH 27
HH 35 (Male)
HH 35 (Female)

Fig. 4 Validation of donor PGC-derived progenies and genome-edited chickens. (**a**) Validation of donor PGC-derived hybrid progenies (*I/I*) compared to endogenous PGC-derived progenies (*I/i*) by feather color. (**b**) Validation of genome-edited chickens with a fluorescent excitation lamp with detection filters. (**c**) Germ cell-specific green fluorescent protein (GFP) expression in an embryonic germinal crescent at HH stage 4 and embryonic gonads at HH stages 27 and 35 of germ cell tracing model. Scale bars, 100 μm. (Figures from Lee et al. [23, 30] and Rengaraj et al. [22])

4 Notes

1. MEFs are isolated at 12.5–13.5 days post coitum (dpc) ICR strain pregnant mice and cultured to P3 at culture dish. Then, the MEFs are treated with mitomycin C at 37 °C for 3 h and frozen in liquid nitrogen. The day before the subculture of PGCs, the mitomycin C-treated MEFs were seeded onto a 0.1% gelatin-coated culture dish with DMEM supplemented with 10% (vol/vol) FBS and 1× ABAM. The optimal confluency of the MEFs is 30–50%.
2. To select genome-edited PGCs, diverse fluorescent reporter genes could be used. For drug selection, apply the optimal

amount of drug after analyzing the kill curve of cultured PGCs with drug-resistant MEFs.

3. T7E1 assay is widely used in the detection of genetic mutations. To identify gene mutation using the T7E1 assay, apply the optimal amount of T7E1 endonuclease and incubate proper times with control. Prolonged incubation or applying an excess amount of enzyme could result in over-digestion.
4. Donor PGC-derived progenies could be distinguishable for feather color because WL feather color (*I/I*) is dominant and Korean Ogye feather color (*i/i*) is recessive. Therefore, recipient Korean Ogye chickens that have donor WL PGCs (*I/I*) and their own PGCs (*i/i*) could produce WL phenotype progenies (*I/i* or *I/I*) through testcross with wild-type Korean Ogye chicken (*i/i*) or WL chicken (*I/I*). The donor PGCs could also be the Korean Ogye breed, too. Recipient WLs that have donor Korean Ogye PGCs (*I/I* and *i/i*) could produce donor Korean Ogye PGC-derived progenies (*i/i*) through testcross with wild-type Korean Ogye chickens (*i/i*).
5. Off-target effect is an unavoidable issue in CRISPR technology. To assess further nonspecific mutation in the gene-targeted chickens, web-based off-target screening and whole genome sequencing (WGS) of the chicken genomic DNA are strongly recommended. Although many software tools provide a list of potential off-target effects for CRISPR technology, however, the predictions with the tools can cause false positives and negatives [34].

Acknowledgments

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Chapter 24

CRISPR-Cas9-Mediated Genome Modifications in Zebrafish

Yusuke Kamachi and Atsuo Kawahara

Abstract

CRISPR-Cas9 genome editing technology has been successfully applied to generate various genetic modifications in zebrafish. The CRISPR-Cas9 system, which originally consisted of three components, CRISPR RNA (crRNA), *trans*-activating crRNA (tracrRNA), and Cas9, efficiently induces DNA double-strand breaks (DSBs) at targeted genomic loci, often resulting in frameshift-mediated target gene disruption (knockout). However, it remains difficult to perform the targeted integration of exogenous DNA fragments (knock-in) with CRISPR-Cas9. DSBs can be restored through DNA repair mechanisms, such as nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homology-directed repair (HDR). One of our two research groups established a method for the precise MMEJ-mediated targeted integrations of exogenous genes containing homologous microhomology sequences flanking a targeted genomic locus in zebrafish. The other group recently developed a method for knocking in ~200 nt sequences encoding composite tags using long single-stranded DNA (ssDNA) donors. This chapter summarizes the CRISPR-Cas9-mediated genome modification strategy in zebrafish.

Key words Zebrafish, CRISPR-Cas9, NHEJ, MMEJ, HDR, Long ssDNA, Knockout, Knock-in

1 Introduction

Zebrafish is an ideal model vertebrate species for forward genetics [1]; therefore, a number of zebrafish mutants with defects in organogenesis have been identified via unbiased chemical mutagenesis screening [2, 3]. Through such screening, the genes responsible for the phenotypes of these mutants have been identified as key regulators of early zebrafish embryogenesis, including hematopoietic and cardiovascular development [4, 5]. Recent advances in genome editing technologies (ZFN, TALEN, and CRISPR-Cas9), which are powerful tools for reverse genetics in various model organisms, have enabled the introduction of locus-specific DSBs that are then repaired by DNA repair mechanisms, such as NHEJ, MMEJ, and HDR [6]. DSBs can generally be repaired by HDR using long fragments homologous to the targeted locus. In the absence of such DNA templates, NHEJ connects the ends of

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broken strands and often leads to insertion and/or deletion (indel) mutations. In an alternative DNA repair mechanism, exposed ends with microhomologous sequences (3–30 bases) at the target site are mutually annealed and repaired by MMEJ, resulting in the production of predictable small deletions. Thus, frameshift-mediated targeted gene disruption can be easily achieved through NHEJ and MMEJ. Interestingly, the injection of either two single guide RNAs (sgRNAs) for a targeted gene together with Cas9 into zebrafish embryos, or multiple crRNAs per gene and tracrRNA together with Cas9 into zebrafish embryos efficiently induce biallelic indel mutations [7–10], resulting in the manifestation of a targeted gene knockout phenotype in F0 embryos (crispant embryos). In fact, Suzuki et al. demonstrated that the disruption of the zebrafish *trrap* (*transformation/transcription domain-associated protein*) gene locus in *trrap*-crispant F0 larvae recapitulated the craniofacial defects observed in *trrap* knockout larvae (Fig. 1) [10].

In zebrafish, the targeted integration of exogenous DNA fragments and genes (knock-in) remains more technically challenging than targeted gene disruption (knockout). Because the frequency of HDR is increased by the generation of site-specific DSBs, the genome editing technologies have been used as a tool for the targeted integration of exogenous genes. Auer et al. have reported the NHEJ-mediated knock-in of a Gal4 driver gene into an eGFP transgene [11]. The authors observed the CRISPR-Cas9-mediated targeted insertion of a donor vector when the vector and the targeted genomic locus were simultaneously cleaved via the same CRISPR-Cas9 system. Two other groups have reported the homology-independent knock-in of a vector containing a CRISPR target site, the hsp70 promoter and reporter/driver genes [12, 13]. NHEJ-mediated reporter gene integration at a targeted locus is a powerful genetic tool allowing the investigation of the real-time reporter expression in heterozygous embryos and target gene loss-of-function phenotypes in homozygous embryos [13].

Hisano et al. have developed a method allowing the MMEJ-mediated knock-in of exogenous reporter genes (Fig. 2) [14]. An eGFP reporter construct containing bait sequences targeted by sgRNA-Cas9 and short homologous sequences (40 bases) flanking the targeted genomic locus was used. The reporter gene was precisely integrated into the targeted genomic locus by MMEJ, suggesting that the MMEJ-mediated knock-in method is useful for precise genome modifications in various model organisms.

More recently, Ranawakage et al. have developed a method for knocking in an ~200 base-pair sequence encoding a composite tag using a combination of the CRISPR-Cas9 ribonucleoprotein complex and lssDNA as a donor template (Fig. 3) [15]. For efficient and precise knock-in, the proper selection of lssDNA strands, 3′

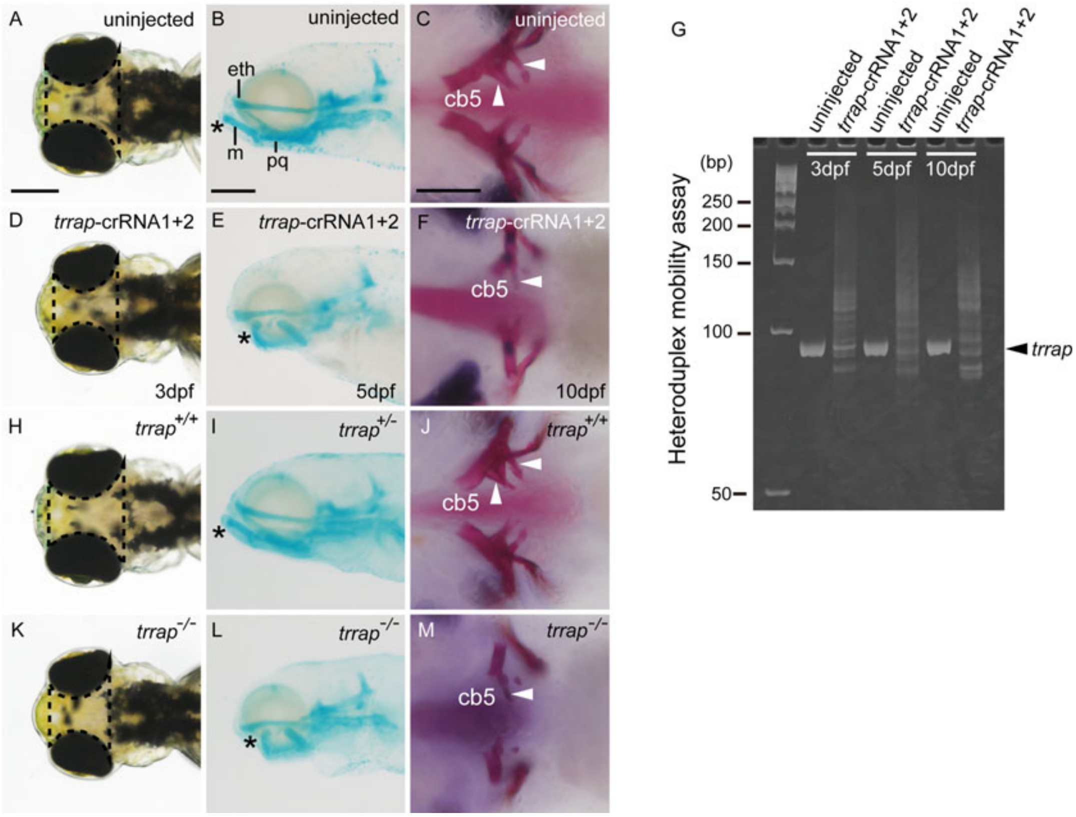

Fig. 1 CRISPR-Cas9-mediated targeted gene disruption in zebrafish. (**a**–**g**) Morphological abnormalities of *trrap*-crispant larvae. As described previously [10], synthetic *trrap*-crRNA1, *trrap*-crRNA2, and tracrRNA were injected with recombinant Cas9 protein into zebrafish embryos. (**a**, **d**) Morphology of the head at 3 dpf. The size of the head of *trrap*-crispant larvae, indicated by the dotted line, is small. Dorsal view. Scale bar, 200 μm. (**b**, **e**) Alcian blue staining of pharyngeal arches at 5 dpf. Lateral view. The morphology of the lower jaw was altered in *trrap*-crispant larvae (asterisk). Eth, ethmoid plate; m, Meckel's cartilage; pq, palatoquadrate. Scale bar, 200 μm. (**c**, **f**) Alizarin red staining of cranial bones at 10 dpf. Ventral view. Tooth mineralization in *trrap*-crispant larvae, indicated by white arrowheads (cb5, ceratobranchial 5), was reduced. Scale bar, 100 μm. (**g**) Various indel mutations were detected at the targeted *trrap* locus of the *trrap*-crispant larvae. (**h**–**m**) Morphological abnormalities of *trrap*-mutant larvae. As described previously [10], the *trrap*-mutant larvae exhibited a small head and morphological defects in the lower jaw and teeth; these morphological abnormalities in the *trrap*-mutants were almost identical to the phenotypes observed in the *trrap*-crispant larvae

homology arm length, and DSB site-to-knock-in site distance has been found to be critical.

This chapter describes the procedures for CRISPR-Cas9-mediated targeted gene disruptions and the CRISPR-Cas9-mediated knock-in of exogenous DNA fragments in zebrafish.

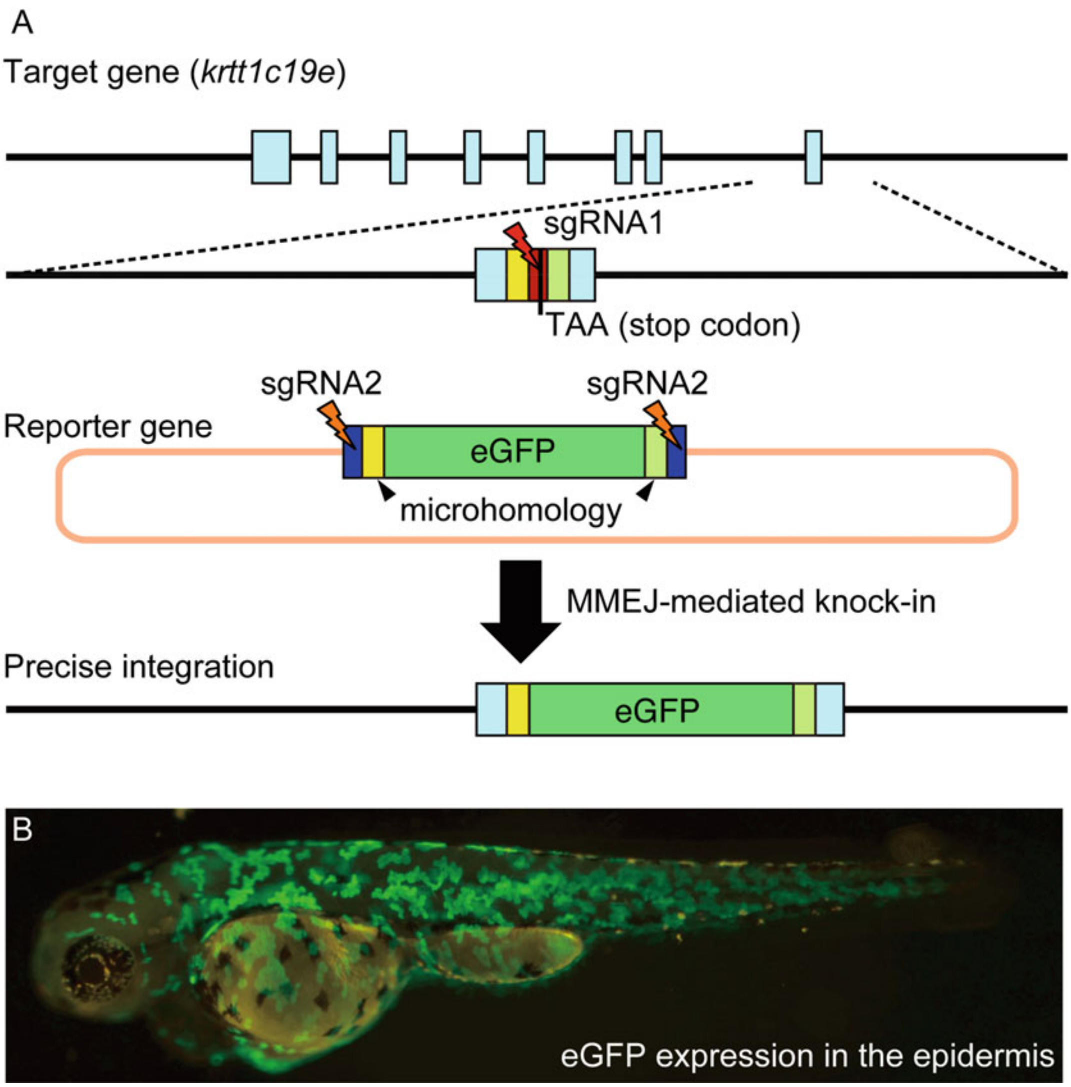

Fig. 2 MMEJ-Mediated knock-in of an exogenous reporter gene. (**a**) Genomic locus of the *krtt1c19e* gene (a *keratin* gene) and the eGFP reporter gene construct. The reporter gene contains two homologous sequences (yellow and light green boxes: 40 bases each) around the stop codon of the *krtt1c19e* gene. Two sgRNAs (sgRNA1, for targeted genome digestion; sgRNA2, for reporter gene digestion) and the reporter gene together with Cas9 mRNA were injected into one-cell stage zebrafish embryos, and a transgenic line containing the eGFP gene at the *krtt1c19e* locus was established. (**b**) eGFP expression in the epidermis at 2 dpf. As described previously [13], eGFP expression was detected in the epidermis of injected embryos

2 Materials

2.1 Preparation of sgRNA, crRNA, tracrRNA, Cas9 mRNA, and Recombinant Cas9 Protein

1. pDR274 (Addgene plasmid number: 42250) [16] (for sgRNA synthesis).
2. pCS2+hSpCas9 (Addgene plasmid number: 51815) [17] (for Cas9 mRNA synthesis) (*see* **Note 1**).

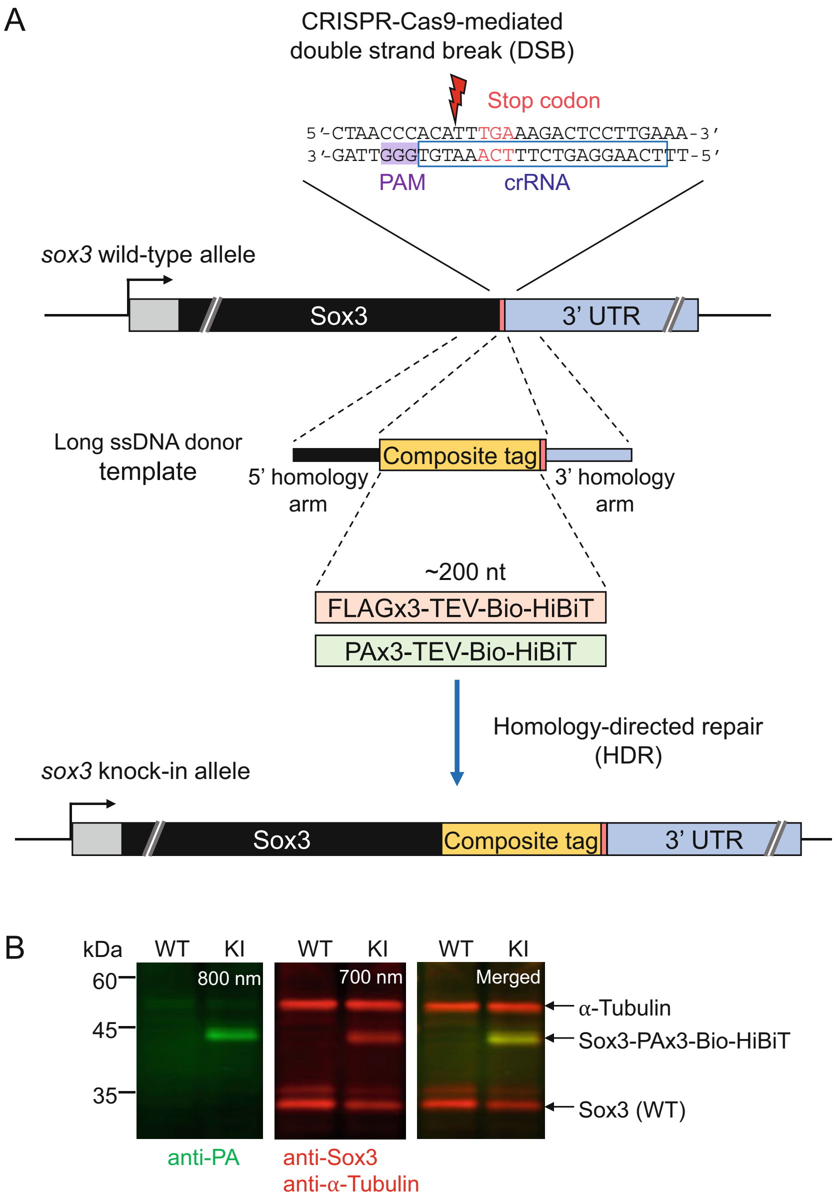

Fig. 3 Knock-in of composite tag-encoding sequences via the CRISPR-Cas9 system. (**a**) An ∼200-nt sequence encoding a composite tag that contains either a FLAGx3 or PAx3 epitope tag followed by a tobacco etch virus (TEV) protease cleavage site, a biotin acceptor domain (Bio tag), and a HiBiT peptide tag was knocked into the 3′ end of the coding sequence of the *sox3* gene [15]. A long ssDNA donor fragment that contains the composite tag flanked at both ends by the homology arms corresponding to the CDS upstream from the stop codon (5′ homology arm) and the 3′ UTR downstream from the stop codon (3′ homology arm) of the *sox3* gene was used as a template to induce the homology-directed repair (HDR) mechanism after CRISPR-Cas9-mediated double-strand break (DSB) generation. (**b**) The tagged Sox3 protein was detected by two-color Western blotting using antibodies against Sox3 and the PA tag. The knock-in (KI) sample was prepared from F1 embryos derived from a cross between an F0 founder with biallelic insertion of the PAx3-Bio-HiBiT tag sequence and wild-type fish. α-Tubulin was used as a loading control

3. Synthetic crRNA (for dual-guide RNAs).
4. Synthetic tracrRNA (for dual-guide RNAs).
5. Recombinant Cas9 protein (for CRISPR-Cas9 ribonucleoprotein injection).
6. *Bsa*I-HF (restriction enzyme).
7. *Dra*I (restriction enzyme).
8. *Not*I-HF (restriction enzyme).
9. 20 mg/mL proteinase K.
10. Phenol/chloroform/isoamyl alcohol (25:24:1).
11. 3 M sodium acetate.
12. 5 M ammonium acetate.
13. 100% ethanol.
14. 70% ethanol.
15. Plasmid purification kit, such as QIAprep Spin Miniprep Kit (QIAGEN).
16. RNA transcription kit, such as MAXIscript T7 Transcription Kit and mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Scientific).
17. G-25 column, such as PD SpinTrap™ G-25 (GE Healthcare Life Sciences).
18. Primer: M13 reverse primer; 5′-CAGGAAACAGCTATGAC-3′.
19. LB medium: 1% polypeptone, 0.5% yeast extract, and 1% NaCl.
20. Antibiotics: kanamycin.
21. Ligase, such as ligation high version 2 (TOYOBO).
22. Competent *E. coli* (DH5α).
23. Ethidium bromide solution (0.5 μg/ml).
24. Magnetic beads, such as NucleoMag NGS Clean-up and Size Select (MACHEREY-NAGEL).

2.2 Preparation of Donor Templates

1. Preparation of long ssDNA using pLSODN-1/2D.
2. Nicking endonucleases (NEB).
3. Denaturing gel loading buffer.
4. DNA Purification kit, such as NucleoSpin Gel and PCR Clean-up columns (MACHEREY-NAGEL).

2.3 Microinjection

1. Injection buffer: 40 mM HEPES [pH 7.4], 240 mM KCl, and 0.5% phenol red.
2. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$.

3. Puller, such as PC-10 (Narishige).
4. Manipulator, such as MMN-8 (Narishige).
5. Microinjector, such as IM 300 (Narishige).

2.4 Genomic PCR and Sequencing Analysis

1. TA-cloning vector, such as pGEM-T Easy (Promega).
2. M13 forward primer, 5′-GTAAAACGACGGCCAGT-3′.
3. PCR product purification kit, such as MinElute PCR Purification Kit.
4. Thermal Cycler, such as Applied Biosystems 2720.

3 Methods

3.1 Construction of sgRNA Expression Vectors

1. Search sgRNA target sequences for individual target loci; the NGG sequence is required for the recognition of the sgRNA-Cas9 complex. Choose a 20-nucleotide sequences upstream of the NGG as a target site. Prepare oligonucleotides encoding the sgRNA target sequences (sense oligonucleotide, 5′-TAGGN$_{18}$-3′, and antisense oligonucleotide, 5′-AAACN$_{18}$-3′) (*see* **Note 2**).
2. Mix sense and antisense oligonucleotides (1 μM each), heat the solution at 98 °C for 3 min, incubate it at 65 °C for 10 min, and leave the solution at room temperature.
3. Mix 1 μL of the annealed oligonucleotides, 1 μL of the *Bsa*I--digested pDR274 vector (10 ng/μL) and 2 μL of ligase, and incubate the mixture at 16 °C for 30 min.
4. Transform the ligation solution (2 μL) into competent *E. coli* (20 μL).
5. Select bacterial colonies containing sgRNA sequences and grow the bacteria in LB medium with kanamycin.
6. Purify the sgRNA expression vector from bacterial cultures by using plasmid purification kit.
7. Read the nucleotide sequences of the inserted fragments by DNA sequencing.

3.2 Preparation of sgRNA

1. Linearize 5 μg of the sgRNA expression vector by *Dra*I digestion.
2. Incubate the linearized DNA with 200 μg/mL proteinase K at 50 °C for 30 min (*see* **Note 3**).
3. Extract the linearized DNA by using phenol/chloroform/isoamyl alcohol (25:24:1).
4. Transfer the supernatant to a 1.5 mL tube, add a one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol, and centrifuge at 15,000 × g for 10 min.

5. Rinse the pellet with 70% ethanol and dry the pellet.
6. Dissolve the pellet in sterilized water.
7. Transcribe sgRNA from the linearized DNA (1 μg) by using RNA transcription kit.
8. Add 1 μL of DNase I, and incubate the mixture at 37 °C for 15 min (*see* **Note 4**).
9. Extract the RNA with phenol/chloroform/isoamyl alcohol (25:24:1).
10. Remove unincorporated nucleotides by gel filtration.
11. Precipitate the sgRNA with a one-tenth volume of 5 M ammonium acetate and two volumes of 100% ethanol.
12. Dissolve the pellet in sterilized water.

3.3 Preparation of *Cas9* mRNA

1. Linearize 5 μg of pCS2+hSpCas9 by *Not*I digestion.
2. Incubate the linearized DNA with 200 μg/mL proteinase K at 50 °C for 30 min to abolish RNase activity.
3. Extract the linearized DNA with phenol/chloroform/isoamyl alcohol (25:24:1).
4. Transfer the supernatant into a 1.5 mL tube, add a one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol, and centrifuge at 15,000 × g.
5. Transcribe mRNA from the linearized DNA (1 μg) by using RNA transcription kit.
6. Follow the same processes (**steps 8–12**) specified under Subheading 3.2.

3.4 Preparation (Construction) of dsDNA Donors

1. Amplify eGFP fragments using primers containing the left or right homology arms (40 nt) for the targeted genomic site (*see* **Note 5**).
2. Insert the amplified eGFP fragments into a donor vector containing one or two bait sequences targeted by a donor-specific gRNA (e.g., eGFP-gRNA).
3. Read the nucleotide sequences of the inserted fragments by DNA sequencing.

3.5 Preparation of lssDNA Donors

1. Amplify the DNA fragments of the left and right homology arms of the donor DNA template from zebrafish genomic DNA using specific primers containing restriction sites; the length of each arm can range from 50 to 300 nt (*see* **Note 5**). Clone these two arm fragments and a DNA fragment encoding an epitope or composite tag into the multicloning sites of pUC19 via quadruple ligation.

2. Subclone the donor sequence assembled in pUC19 at a central position within the multicloning sites of pLSODN-1/2D (e.g., SmaI or EcoRV); it is advisable to obtain both orientations of the insert to isolate and test both strands for knock-in.
3. Digest 100 μg of the resulting plasmids with a combination of two nicking endonucleases or nicking and restriction endonucleases.
4. Precipitate the digested DNAs with ethanol and dissolve them in 50 μl of water. Add 150 μL of denaturing gel loading buffer. After heat denaturation at 70 °C for 5 min, separate lssDNAs using agarose gel electrophoresis. After electrophoresis, stain the gel with the ethidium bromide solution. Excise the bands corresponding to the lssDNA donor fragments and purify them using DNA purification spin columns.

3.6 Microinjection

1. Prepare the injection needle with a puller.
2. For knockout experiments, prepare an injection solution containing the CRISPR-Cas9 components (crRNA-1, 25 ng/μL; crRNA-2, 25 ng/μL; tracrRNA, 100 ng/μL; Cas9 protein, 1 μg/μL in injection buffer) [10]. For knock-in experiments using a dsDNA donor, prepare an injection solution containing sgRNAs, the donor vector, and Cas9 mRNA solutions (sgRNA, 25 ng/μL; donor vector, 25 ng/μL; Cas9 mRNA, 250 ng/μL in injection buffer) [14]; two sgRNAs are used: one for targeted genomic sequence digestion and another for reporter plasmid digestion. For knock-in experiments using an lssDNA donor, prepare the injection solution by mixing the lssDNA solution with the 1.5 μM RNP complex to obtain final concentrations of 0.2 μM lssDNA and 1 μM RNP complex.
3. Fill the needle with the solution.
4. Inject approximately 1 nL of the solution into one-cell stage zebrafish embryos (1.5 nL for the lssDNA injection solution).
5. Incubate the injected embryos in E3 medium at 28.5 °C (*see* **Note 6**).

3.7 Preparation of Genomic DNA

1. Place one zebrafish embryo (1-day postfertilization, dpf) in a 1.5 mL tube and remove as much liquid as possible.
2. Add 108 μL of 50 mM NaOH and incubate the tube at 98 °C for 10 min.
3. Add 12 μL of 1 M Tris–HCl [pH 8.0]; mix the solution and store it at −30 °C.

3.8 Genomic PCR for the Evaluation of gRNA Efficiency

1. It is advisable to determine the genome editing efficiency using a heteroduplex mobility assay (HMA) or Sanger sequencing-based ICE/TIDE tools.
2. Amplify the DNA fragment containing the CRISPR-Cas9 target site from genomic DNAs of edited and wild-type embryos using the locus-specific primers (*see* **Note 7**).
3. For HMA, separate the resultant PCR amplicons on 12.5% polyacrylamide gels (Fig. 1).
4. For an ICE/TIDE analysis, purify the PCR products using magnetic beads or spin columns (*see* Subheading 3.7). Perform Sanger sequence of the PCR products from both the edited and wild-type embryos. Input trace files into the ICE (https://ice.synthego.com) or TIDE (https://tide.nki.nl) online tools to analyze editing efficiency.

3.9 Genomic PCR for the Evaluation of Knock-in Events

1. It is advisable to determine the knock-in efficiency using genomic DNA prepared from the CRISPR-Cas-injected embryos before rearing the injected F0 fish to obtain founders. For F0 founder fish identification, use F1 embryos derived from crosses of F0 fish with wild-type fish.
2. Amplify the DNA fragment containing the junction by genomic PCR using a locus-specific primer and a reporter (or tag)-specific primer (*see* **Note 7**).
3. Add 2 μL of 6× loading dye to the reaction solution and separate PCR products on a 1% agarose gel.
4. Obtain an image of the gel with a transilluminator.
5. Evaluate knock-in efficiency in the F0 embryos or determine the potential founders containing the reporter (or tag).

3.10 Determination of the Integration Site by Sequence Analysis

1. Amplify the junction region from the genomic DNA of F1 embryos derived from crosses of potential F0 founders with wild-type fish by PCR using a locus-specific primer and a reporter-specific primer; F0 embryos can be used to confirm the occurrence of precise knock-in events.
2. Purify the PCR products using the PCR Purification Kit.
3. Insert the purified PCR products into the TA-cloning vector (*see* **Note 8**).
4. Transform the ligation solution (2 μL) into competent *E. coli* (20 μL).
5. Select bacterial colonies containing the reporter sequences by PCR using the M13 forward and reverse primers.
6. Purify the PCR products by using the PCR Purification Kit.

7. Sequence the purified PCR products and identify F0 founders containing the correct knock-in allele.
8. Maintain F1 embryos derived from the F0 founders to obtain adult fish.

4 Notes

1. Various types of sgRNA expression vectors and Cas9 expression vectors are commercially available. It is important to check the genome editing activity of your vector system.
2. The CRISPR-Cas9 target site has the sequence, 5′- N_{20}-NGG-3′. To produce an sgRNA expression vector, NN at the 5′ end of the target sequence can be replaced with a GG sequence because the 5′ end sequence is not essential for sgRNA-Cas9 recognition.
3. Proteinase K is used to abolish RNase activity.
4. DNase I is used to digest the template DNA.
5. The introduction of blocking mutations that prevent cleavage by Cas9 may be required when the homology arm sequences include the intact CRISPR-Cas9 target sequence.
6. The genome editing activity of sgRNA-Cas9 is determined in a heteroduplex mobility assay (HMA) using genomic DNA derived from an injected embryo.
7. Locus-specific primers (approximately 20 bp) are selected by using the primer design tool "Primer3" (http://bioinfo.ut.ee/primer3-0.4.0/). For sgRNA evaluation, design forward and reverse primers located approximately 100 bp (for HMA) or 300 bp (for ICE/TIDE) from the CRISPR-Cas9 target site. Design another internal primer for Sanger sequencing to obtain optimal sequencing quality.
8. The purified PCR products can be directly sequenced, particularly when the PCR is performed using individual F1 embryos.

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Chapter 25

Genome Editing of Medaka

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Abstract

Medaka (*Oryzias latipes*), along with zebrafish (*Danio rerio*), is a useful experimental model fish. Here, we describe a simple method for generating medaka gene knockout strains using an automated microchip electrophoresis system. We also describe a method for targeted gene knockin using a plasmid carrying a sequence that does not cause off-target effects in medaka. Additionally, knockin method without plasmid cloning is described.

Key words Medaka, Genome editing, CRISPR/Cas, Targeted mutagenesis, Heteroduplex mobility assay, Targeted integration

1 Introduction

Medaka (*Oryzias latipes* species complex) is a small freshwater teleost fish native to East Asia and has been used as an experimental vertebrate model since the early twentieth century [1]. In recent years, owing to availability of a number of genetic resources, including highly polymorphic inbred strains, mutants with unique phenotypes, and complete genome sequence, medaka has become an attractive model system for genetic analysis in many areas of biological research [1]. In particular, the availability of genetic resources has contributed to advances in forward genetic studies, such as identification of the sex-determining gene [2] and a novel gene responsible for a human disease [3].

Reverse genetics is another approach used to determine gene function and understand biological processes. Although gene targeting in mouse embryonic stem cells is a powerful tool for reverse genetics, this technique has not been a feasible option yet in medaka because of the difficulty in establishing germline-competent stem cell lines.

Genome editing has become a robust and versatile technology for carrying out reverse genetic studies in a wide range of species,

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including medaka and other fish. In this technology, site-specific DNA double-strand breaks (DSBs) induced by targetable nuclease systems result in targeted genome manipulations, such as targeted mutagenesis with a small insertion and/or deletion (indels) or targeted gene integration, during the subsequent repair processes. RNA-guided nuclease (RGEN) based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is one of the simplest nuclease systems to implement because target specificity of the Cas9 nuclease can be easily tailored by customizing the single guide RNA (sgRNA) or CRISPR RNA (crRNA) sequence.

In this chapter, we describe protocols for targeted mutagenesis and targeted integration in medaka using the CRISPR/Cas system. First, we describe methods to prepare genome editing tools for microinjection, which is commonly used to introduce genome editing tools into fish eggs. Then, we describe heteroduplex mobility assay (HMA) performed with an automated microchip electrophoresis system (MultiNA, HMA-MultiNA), a simple method to detect small indels induced by the RGEN. This method is convenient to evaluate DSB-inducing activity of the sgRNA or crRNA and to identify mutated individuals for targeted mutagenesis experiments. Finally, we describe the procedure for targeted integration using a plasmid that contains a unique target sequence (BaitD sequence) and also using PCR fragments, with which hassle of plasmid cloning process is eliminated [4].

2 Materials

2.1 Preparation of Single-Guide RNA (sgRNA)

This section describes protocol for producing sgRNA with PCR-amplified template. Here, the protocol of *CUGA*®7 gRNA Synthesis Kit (NIPPON GENE, Japan) is described, but, of course, there is no problem using equivalent kits supplied by other suppliers. This kit requires three kinds of oligonucleotide DNA: Oligo A containing T7 promoter, 20 bp of the targeted sequence, and part of crRNA/tracrRNA sequence. Oligos B and C contain crRNA/tracrRNA sequence. These oligos are used for PCR and produce 119 bp fragments, which are used for the later transcription:

1. CRISPR/Cas9 target online predictor such as CRISPRdirect (https://crispr.dbcls.jp/) [5] or CRISPRscan (http://www.crisprscan.org/) [6].
2. NBRP (National Bio Resource Project) medaka pattern match tool for CRISPR/Cas (http://viewer.shigen.info/medakavw/crisprtool/).
3. Search for CRISPR target sites with microhomology sequences (http://viewer.shigen.info/cgi-bin/crispr/crispr.cgi).

4. Three kinds of oligonucleotide DNA: Oligo A (5′-CTAATACGACTCACTATAG(X_{20})GTTTTAGAGCTAGAAATAGCA-3′, where X_{20} is 20 nucleotide sequence of target locating upstream of PAM), Oligo B (5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3′), and Oligo C (5′-AAAAGCACCGACTCGGTGCC-3′) containing crRNA/tracrRNA sequence (*see* Subheading 3.1, **steps 1–3**).
5. DNA purification kit.
6. 20 mg/mL proteinase K solution.
7. 10% SDS solution.
8. *CUGA*®7 gRNA Synthesis Kit (NIPPON GENE, Japan).
9. RNA extraction kit.

2.2 Production of Cas9 mRNA

1. pCS2+hSpCas9 (Addgene Plasmid #51815).
2. Plasmid purification kit.
3. 20 mg/mL proteinase K solution.
4. 10% SDS solution.
5. SP6 RNA polymerase transcription kit.
6. RNA extraction kit.

2.3 Detection of Mutations in Injected Embryos and Fin Clips Using Heteroduplex Mobility Assay with an Automated Microchip Electrophoresis System (HMA-MultiNA)

1. Alkaline lysis buffer: 25 mM NaOH and 0.2 mM EDTA.

   Prepare fresh by diluting 1 M NaOH (40× stock solution) and 10 mM EDTA (50× stock solution).
2. Neutralization buffer: 40 mM Tris–HCl (pH 8.0).
3. High success-rate DNA polymerase such as KOD-FX (Toyobo, Osaka, Japan).
4. Gene-specific primers: a primer pair that can amplify an 80–250 bp genomic DNA fragment containing the target site (*see* **Note 1**).
5. Thermal cycler.
6. MCE-202 MultiNA microchip electrophoresis system for DNA/RNA analysis with DNA-500 reagent kit for MultiNA (Shimadzu, Kyoto, Japan).

2.4 Microinjection into Fertilized Eggs

1. Medaka eggs within 20 min postfertilization (*see* **Note 2**).
2. Plastic dishes (30 mm diameter).
3. Ice or a refrigerated chamber.
4. Micromanipulator.
5. Injector.

6. Stereomicroscope.
7. Egg holder.

2.5 Evaluation of Guide RNA (gRNA) Activity

1. 25 ~ 50 ng/μL sgRNAs (*see* Subheading 2.1) or 10–25 ng/μL crRNA and 40 ng/μL tracrRNA (*see* **Note 3**).
2. 100–200 ng/μL Cas9 mRNA (*see* Subheading 2.2).
3. Medaka eggs earlier than 20 min postfertilization.
4. Microinjection apparatus (*see* Subheading 2.4).
5. Materials for HMA-MultiNA (*see* Subheading 2.3).

2.6 Establishment of Gene Knockout (KO) Strains

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 25 ng/μL sgRNAs (selected in Subheading 2.5).
4. 100–200 ng/μL Cas9 mRNA (*see* Subheading 2.2).
5. Microinjection apparatus (*see* Subheading 2.4).
6. Materials for HMA-MultiNA (*see* Subheading 2.3).

2.7 Preparation of Donor Plasmids and Single-Guide RNAs for Targeted Integration

1. Donor plasmid (pBaitD or p2BaitD): these plasmids contain bait (BaitD) sequences (5′- CCTCGCAGTCTAGGCCGAAGATC-3′) and a multiple cloning site (MCS) (Fig. 1a) (*see* **Note 4**). For knockin with Cas9 nickase, use p2BaitD which contain two pairs of inverted repeats of BaitD sequences (*see* **Note 5**).
2. Two pairs of gene-specific primers: primer pairs that amplify about 500 bp of upstream (left homology arm, HA-L) and downstream (right homology arm, HA-R) regions of the targeted integration site with the appropriate restriction enzyme sites to construct a donor plasmid (*see* Fig. 1b, c for restriction enzyme sites) (*see* **Note 6**).
3. PCR fragment containing reporter gene sequence and polyadenylation site with BamHI and EcoRI recognition sites (*see* **Note 6**).
4. Restriction enzymes XhoI.
5. Restriction enzyme BamHI.
6. Restriction enzyme EcoRI.
7. Restriction enzyme SpeI.
8. Plasmid purification kit.
9. 20 mg/mL proteinase K solution.
10. 10% SDS solution.
11. DNA purification kit.

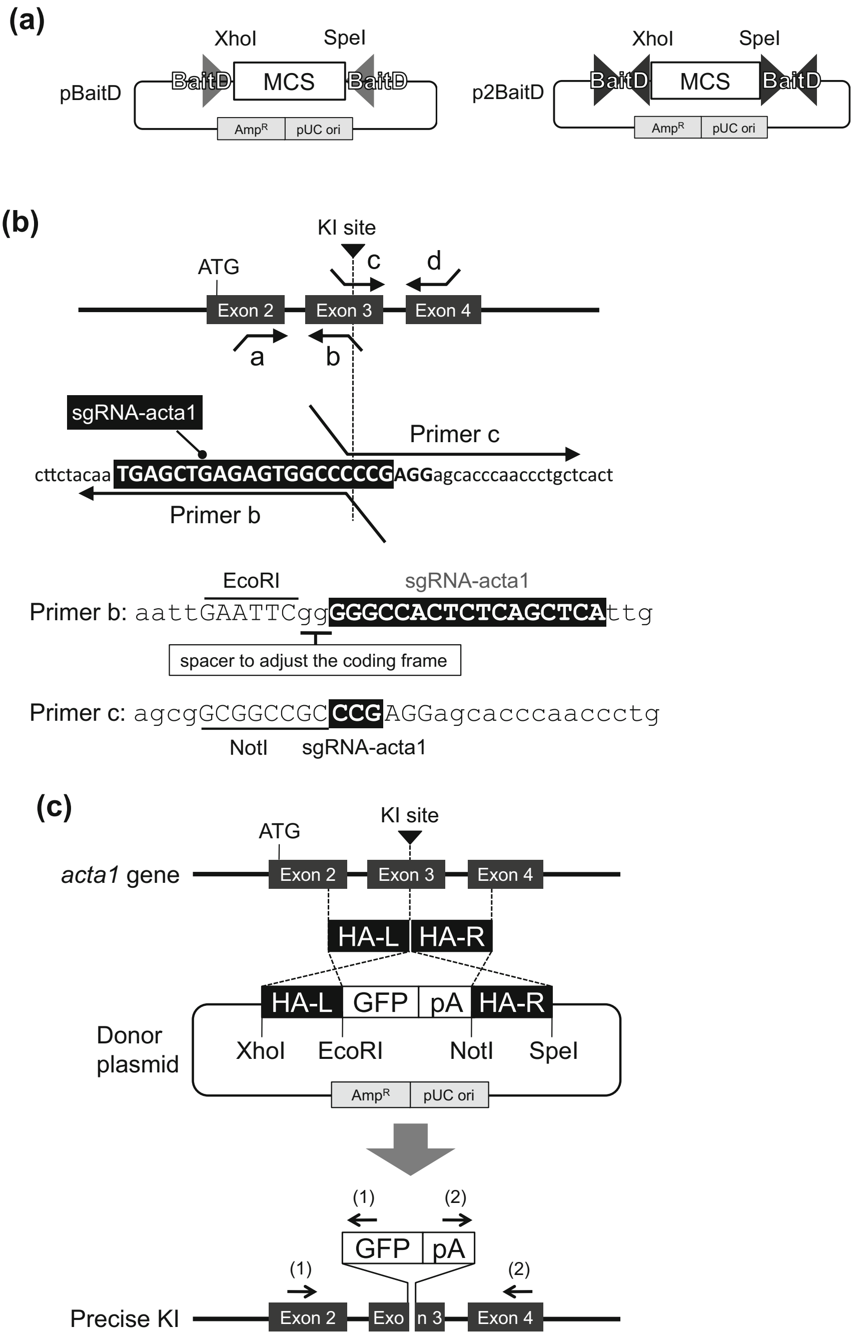

Fig. 1 Schematic illustration of a gene knockin (KI) experiment. (**a**) Schematic design of the pBait D and p2BaitD plasmid. The multiple cloning site (MCS) contains XhoI and SpeI recognition sequences. (**b**) Design of primers to construct homology arms of a donor vector. Primer pairs a/b and c/d can be used to amplify the left homology arm (HA-L) and the right homology arm (HA-R), respectively. A target site of sgRNA for the *acta1* gene (sgRNA-acta1) is indicated in uppercase characters within a gray box. Oligonucleotide sequences of primers b and c are shown below the panel. Two nucleotides, “GG,” are incorporated in primer b to adjust the coding frame. (**c**) Schematic design of a donor vector for precise integration of *GFP* gene in the muscle actin gene (*acta1*) locus. Arrows labeled (1) and (2) indicate primer pairs for PCR genotyping to detect precise integration

12. Three kinds of oligonucleotide DNA: Oligo A-X containing 20 bp of the targeted sequence and oligos B and C containing crRNA/tracrRNA sequence (*see* Subheading 3.1, **steps 1–3**).
13. *CUGA*®7 gRNA Synthesis Kit (NIPPON GENE, Japan).
14. RNA extraction kit.

2.8 Establishment of Gene Knockin (KI) Strains with Cas9 Protein

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 50 ng/μL sgRNA for BaitD.
4. 25 ng/μL sgRNA for the targeted gene.
5. 100–200 ng/μL Cas9 mRNA.
6. 2.5 ng/μL donor plasmid and sgRNAs (*see* Subheading 2.7).
7. Microinjection apparatus (*see* Subheading 2.4).

2.9 Establishment of Gene Knockin (KI) Strains with Cas9 D10A Nickase Protein

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 50 ng/μL sgRNA for BaitD.
4. 25 ng/μL sgRNA for the targeted gene.
5. 1000 ng/μL Cas9 D10A nickase protein.
6. 2.5 ng/μL donor plasmid and sgRNAs (*see* Subheading 2.7).
7. Microinjection apparatus (*see* Subheading 2.4).
8. pCS2+Cas9-mSA (Addgene Plasmid #103882).

2.10 Establishment of Gene Knockin (KI) Strains with PCR Fragment

Recent research showed another gene knockin method without plasmid cloning [4]. This method employs a PCR fragment as a donor, including short homology arms of 30–40 bp at both ends. Originally, this method was done in mice, with monomeric streptavidin-tagged Cas9 mRNA and the biotinylated donor PCR fragment. Although this tagging raised the knockin efficiency in mice [7], the knockin efficiency was comparable with and without tagging in medaka [4]:

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 15–20 ng/μL sgRNA for the targeted gene.
4. 150 ng/μL streptavidin-tagged Cas9 (Cas9-mSA) mRNA.
5. 8–10 ng/μL biotinylated PCR fragments which include 30 bp of homology arms.
6. Microinjection apparatus (*see* Subheading 2.4).

3 Methods

3.1 Preparation of Single-Guide (sgRNA)

1. Select “5′-N_{21}GG-3′” or “5′-CCN_{21}-3′” sequences in loci that are to be targeted. Choose two or more target sites in the target locus with reference to (a) potential off-target sequences and/or (b) microhomology sequences:
    (a) Some genome sequences containing up to several base pairs of mismatches adjacent to a NGG protospacer adjacent motif (PAM) can be disrupted in the injected fish [8]. Target candidates with less potential off-target sites in the medaka genome can be predicted using the CRISPR/Cas9 target online predictor. Potential off-target sites in the medaka genome can also be found using the NBRP medaka pattern match tool for CRISPR/Cas.
    (b) We previously demonstrated that microhomologous sequences that stride across the DSB point frequently induce specific patterns of deletions between two homologous sequences [9]. Candidates that will frequently produce specific patterns of deletions in the RNA-injected fish can be identified using the program hosted at the NBRP medaka website to search for CRISPR target sites with microhomology sequences.
2. Order and obtain three kinds of oligonucleotide DNA from a supplier of choice: Oligo A containing 20 bp of the targeted sequence and Oligos B and C containing crRNA/tracrRNA sequence.
3. Amplify the template DNA using three oligonucleotides: Oligo A (including target 20 bp sequence), Oligo B, and Oligo C, for the sgRNA synthesis. The PCR programs were as follows: one cycle at 94 °C for 2 min followed by 35 PCR cycles of 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 5 s.
4. Add 5 μL of 10% SDS and 2 μL of proteinase K solution to the resulting PCR products, and incubate at 55 °C for 30 min to eliminate residual RNase activity (*see* **Note 7**). Then purify the products using DNA purification kit.
5. Transcribe in vitro with RNA synthesis kit such as *CUGA*®7 gRNA Synthesis Kit following the manufacturer’s protocol.
6. Purify all synthesized sgRNAs using RNA extraction kit (*see* **Note 8**). Store sgRNA at −80 °C.

This is the new cloning-free protocol to prepare sgRNA. For conventional protocol using DR274 vector, refer to [8].

3.2 Production of Cas9 mRNA

1. Purify pCS2+hSpCas9 vector from cultured *E. coli* using plasmid purification kit. Linearize 10 μg of the purified plasmid by NotI digestion in reaction volume of 100 μL.

2. Add 5 μL of 10% SDS and 1 μL of proteinase K solution to the NotI-digested plasmid and incubate at 55 °C for 30 min. Purify the digested plasmid using phenol/chloroform extraction followed by ethanol precipitation (*see* **Note 7**). Dissolve the pellet in 10–20 μL of RNase-free water. This solution can be stored at −20 °C.
3. Assemble 9 μL of reaction solution for in vitro transcription by SP6 RNA polymerase transcription kit in a 0.2 mL PCR tube. Add 1 μL of NotI-digested template DNA and incubate at 37 °C for 3–4 h in a thermal cycler.
4. Purify the transcribed RNA solution using RNA extraction kit. Elute the RNA with 30 μL of RNase-free water (*see* **Note 8**). Use 1 μL of the eluted RNA solution to estimate concentration using a spectrophotometer and for carrying out electrophoresis to assess quality of the RNA. Store the transcribed RNA at −80 °C.

3.3 Detection of Mutations in Injected Embryos and Fin Clips Using Heteroduplex Mobility Assay with an Automated Microchip Electrophoresis System (HMA-MultiNA)

1. Extract crude genomic DNA (gDNA) from embryos or fin clips as follows. Place each embryo or fin clip in a 0.2 mL PCR tube, and add 25 μL of alkaline lysis buffer. In case of embryos, the egg envelope should be broken using a forceps or a truncated 2–200 μL micropipette tip (*see* **Note 9** and Fig. 2). Incubate at 95 °C with frequent vortexing until the embryo or fin clip is dissolved (*see* **Note 10**). Now, add 25 μL of neutralization buffer. These crude DNA samples can be stored at −20 °C. The fish whose tail fin had been clipped can be bred for more than 2 weeks in a plastic cup (Fig. 2).
2. Assemble 10 μL of PCR reaction mixture using primer pairs designed for each target site. Amplify the genomic region containing the target site of the injected guide RNA using standard PCR conditions.
3. Analyze the PCR product using automatic electrophoresis system MultiNA along with DNA-500 reagent kit.

3.4 Microinjection into Fertilized Eggs

1. Collect eggs within 20 min postfertilization in a plastic dish (*see* [10]).
2. Remove attaching filaments of the eggs using forceps, and place the plastic dish containing the eggs on a sheet of paper placed on ice or in a refrigerated chamber (*see* **Note 11**).
3. Inject the mixture of genome editing tools into the cytoplasm of one- or two-cell stage eggs (*see* **Note 12**). It is recommended that the injection be performed with earlier stage (one-cell stage) eggs. Do not inject into the yolk sac. The volume of injected mixture should be less than 4 nL, as a larger volume will kill the embryos.

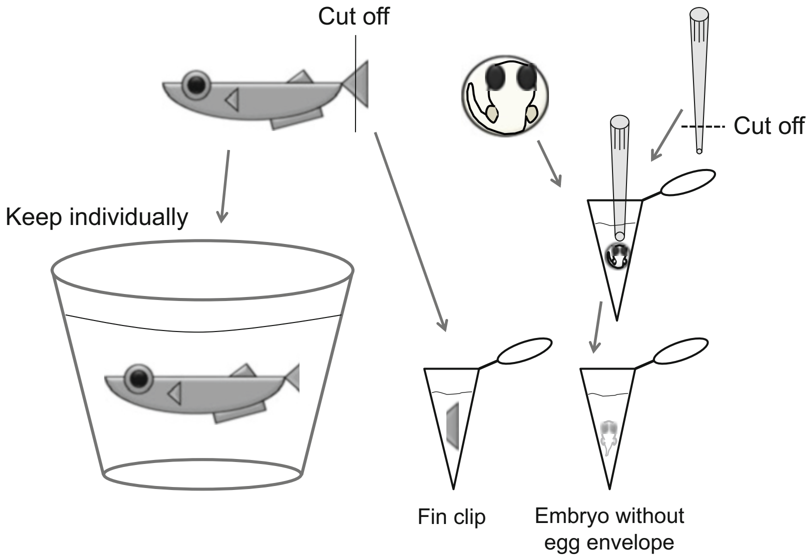

Fig. 2 Fin clip and egg treatment for genomic DNA extraction. Cut off less than each half of tail fin (fin clip) and place it in a 0.2 mL PCR tube containing 25 μL of lysis buffer. The fish is bred in a plastic cup. To break the egg envelope, put an embryo into a 0.2 mL PCR tube containing lysis buffer, and squash it with a truncated 2–200 μL micropipette tip

3.5 Evaluation of Guide RNA (gRNA) Activity

1. Prepare the mixture containing Cas9 mRNA and each designed gRNA. The final concentration of each RNA solution should be as follows: 25–50 ng/μL for sgRNA, 10–25 ng/μL for crRNA, 40 ng/μL for tracrRNA, and 100–200 ng/μL for Cas9 mRNA.
2. Inject each sgRNA or crRNA and tracrRNA with Cas9 mRNA mixture into the fertilized eggs (*see* Subheading 3.4).
3. Incubate injected eggs for 3–5 days at 26–28 °C.
4. Analyze the target region using HMA-MultiNA (*see* Subheading 3.3).
5. Evaluate in vivo activity of gRNAs. Embryos injected with active gRNAs show a number of bands with different mobilities, which are derived from many types of homo- and heteroduplexes containing the wild type and/or various types of indel sequences. As shown in Fig. 3, gRNA with high DSB-inducing activity leads to weak PCR amplification of wild-type sequence (arrowhead).
6. Select the gRNA(s) for the following experiments.

3.6 Establishment of Gene Knockout (KO) Strains

1. Prepare mixture containing 100–200 ng/μL Cas9 mRNA and 25 ng/μL selected gRNA(s) (*see* Subheading 3.5).
2. Inject the mixture into fertilized eggs (*see* Subheading 3.4) (**Note 13**).

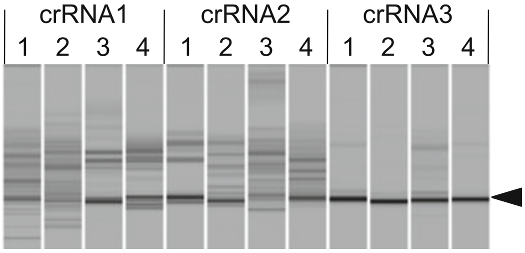

Fig. 3 DNA double-strand break (DSB)-inducing activity of each CRISPR RNA (crRNA). Three crRNAs are designed for a target gene. The in vivo DSB-inducing activity of each crRNA was evaluated using a heteroduplex mobility assay (HMA-MultiNA). crRNA1 and crRNA2 showed high activity, while crRNA3 showed low activity. Black arrowhead indicates the PCR product obtained using wild-type sequence as template. Activity of sgRNAs is evaluated using the same method

3. Incubate the injected embryos at 26–28 °C and rear them until they reach sexual maturity (G_0 adults).
4. Mate the G_0 adult fish with their wild-type counterparts.
5. Collect 8–16 F_1 embryos. Extract gDNA from each embryo and analyze the mutation in gRNA target region using HMA-MultiNA (*see* Subheading 3.3).
6. Directly sequence the PCR fragments showing multiple band patterns in HMA.
7. Select G_0 individuals that produce desired mutant sequences in their progenies (F_1), and obtain their F_1 fish by mating with wild-type fish (Fig. 4a) (*see* **Note 14**).
8. After the F_1 have attained body length > 1 cm, cut off the tail fin from each F_1 fish (Fig. 2).
9. Extract gDNA from each clipped fin and carry out HMA-MultiNA (*see* Subheading 3.3). Determine genotypes by direct sequencing.
10. Select both male and female fish harboring the same desired mutation (Fig. 4b).
11. Mate selected F_1 fish to obtain F_2 fish, and rear them until they have attained body length > 1 cm (*see* **Note 15**).
12. Cut off the tail fin from each F_2 fish. Perform HMA-MultiNA. Heterozygotes show multiple banding patterns in HMA. Though both wild-type and homozygous mutant (KO) fish show single band in HMA, the bands derived from wild-type or KO fish can generally be distinguished according to their size using gel electrophoresis (Fig. 4c).
13. (Optional: for reliable distinction between wild-type and KO fish) To a PCR product showing a single band pattern in HMA, add a PCR product separately prepared from wild-type

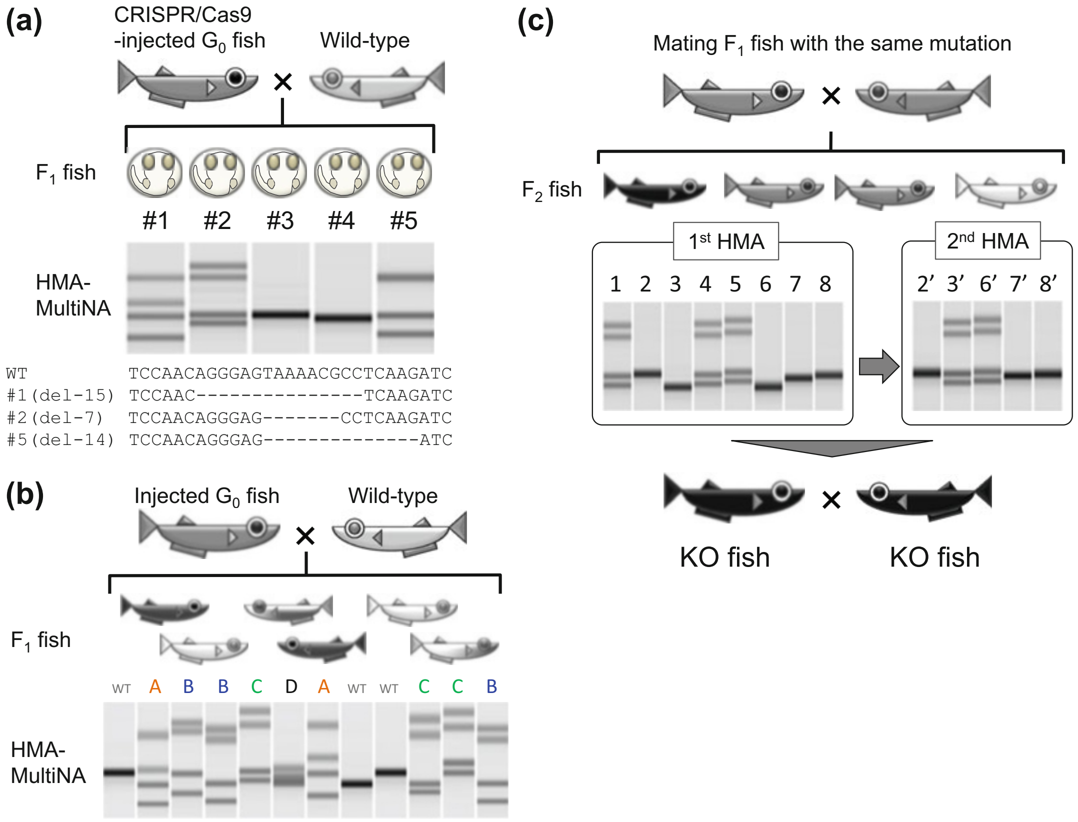
(a)
CRISPR/Cas9 -injected G0 fish
Wild-type
F1 fish
#1 #2 #3 #4 #5
HMA-MultiNA
WT TCCAACAGGGAGTAAAACGCCTCAAGATC
#1(del-15) TCCAAC---------------TCAAGATC
#2(del-7) TCCAACAGGGAG-------CCTCAAGATC
#5(del-14) TCCAACAGGGAG--------------ATC
(b)
Injected G0 fish
Wild-type
F1 fish
WT A B B C D A WT WT C C B
HMA-MultiNA
(c)
Mating F1 fish with the same mutation
F2 fish
1st HMA
1 2 3 4 5 6 7 8
2nd HMA
2′ 3′ 6′ 7′ 8′
KO fish
KO fish

Fig. 4 Heteroduplex mobility assay with an automated microchip electrophoresis system (HMA-MultiNA) to establish gene knockout (KO) strains. (**a**) Selection of G_0 founder fish. Based on HMA-MultiNA and sequencing data of F_1 embryos, select the founder fish that can generate the F_1 generation with desired mutations. In order to establish gene KO fish, frameshift mutations (#2 and #5) are required. (**b**) Selection of F_1 founder fish to produce F_2 generation. HMA-MultiNA genotyping is performed using genomic DNA extracted from a fin clip of each F_1 fish. The HMA profiles indicate the pattern of mutated sequences. A selected fish is then mated with a fish harboring the same mutation pattern to produce the F_2 generation. (**c**) Identification of gene KO fish in a F_2 family obtained by mating F_1 fish harboring the same mutation with each other. Wild-type (2, 7, and 8), heterozygous mutant (1, 4, and 5), and homozygous mutant (3 and 6) fish are distinguished by HMA-MultiNA genotyping using genomic DNA extracted from each fin clip of F_2 fish (first HMA). To clearly discriminate homozygous mutants from wild-type fish, each PCR product from the first HMA is mixed with a wild-type PCR product, and the mixture is reannealed. The PCR products from homozygous mutant fish (3′ and 6′) show a heterozygous pattern (second HMA)

template. Reanneal the mixture by heating at 95 °C for 5 min and gently cooling to room temperature. Perform HMA-MultiNA (second HMA). The wild-type fish will show only one band, while KO fish will show a multiple banding pattern similar to heterozygotes (Fig. 4c).

3.7 Preparation of Donor Plasmids for Targeted Integration

10. Clone PCR fragments containing homologous genomic sequences on either sides of a KI target site (HA-L and HA-R, *see* Fig. 1c) and reporter gene if needed (including polyadenylation site), in the pBaitD or p2BaitD plasmids using restriction enzymes. These plasmids are designated as the donor plasmid (Fig. 1c).
11. Transform *E. coli* with the donor plasmid and culture the *E. coli*.
12. Purify the plasmid from the *E. coli* culture using a plasmid purification kit. Adjust the volume of plasmid solution to 100 μL using sterile water.
13. Add 5 μL of 10% SDS and 1 μL of proteinase K solution in the plasmid solution and incubate at 55 °C for 30 min.
14. Purify the SDS/proteinase K-treated plasmid using DNA purification kit.

3.8 Establishment of Gene Knockin (KI) Strains with Cas9 Protein

For knockin with Cas9 nickase, use 1000 ng/μL nickase protein in place of Cas9 mRNA:

1. Prepare injection mixture containing the following components: 100–200 ng/μL Cas9 mRNA, 25 ng/μL sgRNA-targeted gene, 50 ng/μL sgRNA-BaitD, and 2.5 ng/μL donor plasmid.
2. Inject the mixture into fertilized eggs (*see* Subheading 3.4 and **Note 16**).
3. Incubate the injected embryos at 26–28 °C and rear them to sexual maturity (2–3 months). Figure 5 shows a G_0 embryo in which a precise integration event had occurred in a part of the skeletal muscle.

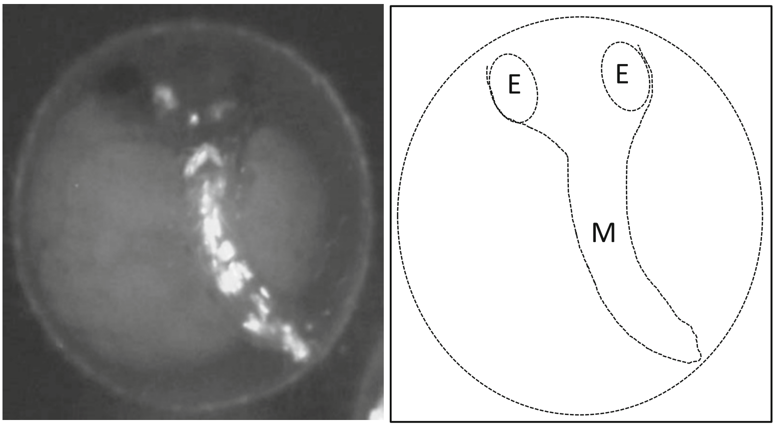

Fig. 5 Precise targeted integration of *GFP* gene into the third exon of the muscle actin (*acta1*) gene. Green fluorescence is observed in the skeletal muscle of an injected embryo 5 days postfertilization. E, eye; M, skeletal muscle

4. Mate the G_0 fish with their wild-type counterparts.
5. Collect 8–16 F_1 embryos and extract gDNA from each embryo (*see* Subheading 3.3).
6. Confirm the precise KI event by PCR genotyping. The primer pairs for this analysis are indicated in Fig. 1c.
7. Select G_0 individuals that produce embryos with the precise KI fragment in their progenies (F_1 generation), and obtain the F_1 fish by mating with their wild-type counterparts.
8. After the F_1 fishes attain body length > 1 cm, cut off the tail fin from each F_1 fish and select KI fish to establish KI strains.

3.9 Establishment of Gene Knockin (KI) Strains with PCR Fragment

1. For Cas9-mSA mRNA synthesis, use pCS2+Cas9-mSA instead of pCS2+hspCas9, and follow Subheading 3.2.
2. Amplify the donor biotinylated PCR fragments as follows: Amplify PCR products with biotinylated primers consisting of 30–40 bp homology arms, with a template plasmid containing reporter gene. Then purify the products with DNA purification kit.
3. Prepare injection mixture containing the following components: 100–200 ng/μL Cas9-mSA mRNA, 25 ng/μL sgRNA-targeted gene, 50 ng/μL sgRNA-BaitD, and 2.5 ng/μL donor plasmid.
4. Follow Subheading 3.8, from **step 2**.

4 Notes

1. Fragments >250 bp lead to decreased resolution of mobility difference among homoduplexes and heterodupulexes.
2. One-cell stage eggs are recommended. Two-cell stage eggs can also be used, but eggs later than the four-cell stage are not recommended because the ratio of mutated cells in an embryo decreases.
3. Usually crRNA and tracrRNA are chemically synthesized. Order and obtain them from a supplier of choice.
4. BaitD sequence was selected from the candidates of highly efficient sgRNA in mammalian cells [11]. Among candidates of the bait sequence, BaitD was the least off-target producing candidate by in silico screening for the reference genomes of 12 fish species.
5. Since Cas9 nickase can only make single-strand breaks, it can only linearize p2BaitD plasmid, but not pBaitD. Cas9 nickase-based knockin is caused by homology-directed repair, so it enables reduction of the DSB-based unwanted mutagenesis at

both on- and off-target sites, caused from nonhomologous end joining [12]. Therefore, Cas9 nickase is recommended to be used in knockin of lethal genes [12].

6. To produce a fusion protein by targeted integration, the primers' sequences should be designed such that the integrated gene fragment (e.g., *GFP* gene) is in-frame with the target native gene.
7. RNase contamination drastically reduces the yield of RNA and success rate of genome editing. To remove RNase activity, the SDS/proteinase K treatment described here is strongly recommended. Alternatively, plasmid preparation using a RNase-free plasmid preparation kit is recommended.
8. RNA extraction kit without DNase is recommended, but removal of the template plasmid DNA by standard DNase treatment can also be carried out.
9. The front ends of commercially available 2–200 uL tips are too narrow to break the egg envelope of medaka. Therefore, cut off the front ends to obtain ~1 mm diameter orifices.
10. Usually, it takes ~5 min to dissolve the fin clip or the embryo.
11. By cooling the fertilized eggs, the one-cell stage period can be extended. However, too low a temperature kills the eggs. Therefore, a 6 °C temperature is recommended. Placing the plastic dish directly on ice decreases the temperature below the recommended one; to avoid this, put a sheet of paper between the plastic dish and ice.
12. It is recommended that the mixture be injected into the cytoplasm of earlier stage (one-cell stage) eggs. Do not inject into the yolk sac. The volume of injected mixture should be less than 4 nL, as a larger volume will kill the embryos.
13. Injection into 30–50 eggs is enough to establish a gene KO strain.
14. To disrupt the gene of interest, G_0 founder fish that generate frameshift mutants in the F_1 generation should be selected.
15. The F_2 generation consists of wild type, heterozygotes, and homozygotes for the mutated sequence in the ratio 1:2:1.
16. Injection into >200 eggs is recommended to establish a gene KI strain.

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Chapter 26

CRISPR-Cas9-Based Functional Analysis in Amphibians: *Xenopus laevis*, *Xenopus tropicalis*, and *Pleurodeles waltl*

Miyuki Suzuki, Midori Iida, Toshinori Hayashi, and Ken-ichi T. Suzuki

Abstract

Amphibians have made many fundamental contributions to our knowledge, from basic biology to biomedical research on human diseases. Current genome editing tools based on the CRISPR-Cas system enable us to perform gene functional analysis in vivo, even in non-model organisms. We introduce here a highly efficient and easy protocol for gene knockout, which can be used in three different amphibians seamlessly: *Xenopus laevis*, *Xenopus tropicalis*, and *Pleurodeles waltl*. As it utilizes Cas9 ribonucleoprotein complex (RNP) for injection, this cloning-free method enables researchers to obtain founder embryos with a nearly complete knockout phenotype within a week. To evaluate somatic mutation rate and its correlation to the phenotype of a Cas9 RNP-injected embryo (crispant), we also present accurate and cost-effective genotyping methods using pooled amplicon-sequencing and a user-friendly web-based tool.

Key words Frog, Newt, Knockout, CRISPR-Cas, Amplicon-sequencing, HMA

1 Introduction

Amphibians have been used in various research fields, developmental and cell biology, genomics, physiology, toxicology, evolution, and human disease models, from the beginning of experimental biology. In particular, metamorphosis and regeneration are unique biological topics in amphibians [1–6]. The African clawed frog (*Xenopus laevis*) has been used most commonly in the world, having a low rearing cost compared to other tetrapod model organisms and with variable mutant and transgenic lines available [7–9]. The Western clawed frog (*Xenopus tropicalis*) has a complete diploid genome, a shorter maturation time than *X. laevis* and 1700 conserved human disease-related genes [10, 11]. The Iberian ribbed newt (*Pleurodeles waltl*) is an emerging model organism that has a relatively short life cycle compared to other species of salamanders [12] and has full regeneration capacity throughout its

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life in multiple organs: limb [13–15], heart [16], jaw, brain [17, 18], and testis [19].

Next-generation sequencing (NGS) and genome editing tools have made a drastic paradigm shift in life science. “Reading” a genome by NGS and “editing” it by CRISPR-Cas make it possible to evaluate gene function in vivo, in ideally all organisms, including non-model organisms. Genome editing tools such as ZFNs, TALENs, and the CRISPR-Cas system can recognize a target sequence and induce DNA double-strand breaks (DSBs). Most of the DSBs are repaired by nonhomologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) and often induce small insertions or deletions (indels) at the target site. Consequently, the target gene is knocked out by frameshift (out-of-frame) or nonsense mutations. CRISPR-Cas is widely used in various organisms including amphibians [15, 20–25]. Cas9 ribonucleoprotein complex (RNP)-injected founder embryos show highly efficient mutation rates and are called “crispants” [1, 26]. The crispant assay facilitates rapid gene functional analysis in non-model organisms in which it is difficult to get the F1 generation and is also commonly accepted even in zebrafish which have a short life cycle.

In this section, we will introduce a workflow of an efficient crispant assay which can be accepted seamlessly for three different amphibians: *X. laevis*, *X. tropicalis*, and *P. waltl*. Researchers can get crispants quite easily by injecting Cas9 protein and in vitro synthesized sgRNAs into a one-cell stage embryo, within a week, without cloning. Since it enables rapid and efficient analysis of genes of interest, we are confident that this workflow will become a major strategy for gene functional analysis in amphibians.

2 Materials

2.1 sgRNA Synthesis

1. Forward oligonucleotide.

   (5′-TAATACGACTCACTATAGG(N)$_{18}$GTTTTAGAGCTAGAAATAGCAAG-3′). (N)$_{18}$ is 18 nucleotides upstream of the protospacer adjacent motif (PAM) for each target site. GG are the first and second nucleotides for T7 RNA polymerase-based in vitro transcription (*see* **Notes 1** and **2** and Fig. 1). The forward oligonucleotides are required for each target site.

2. Reverse oligonucleotide.

   (5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3′). The reverse oligonucleotide contains

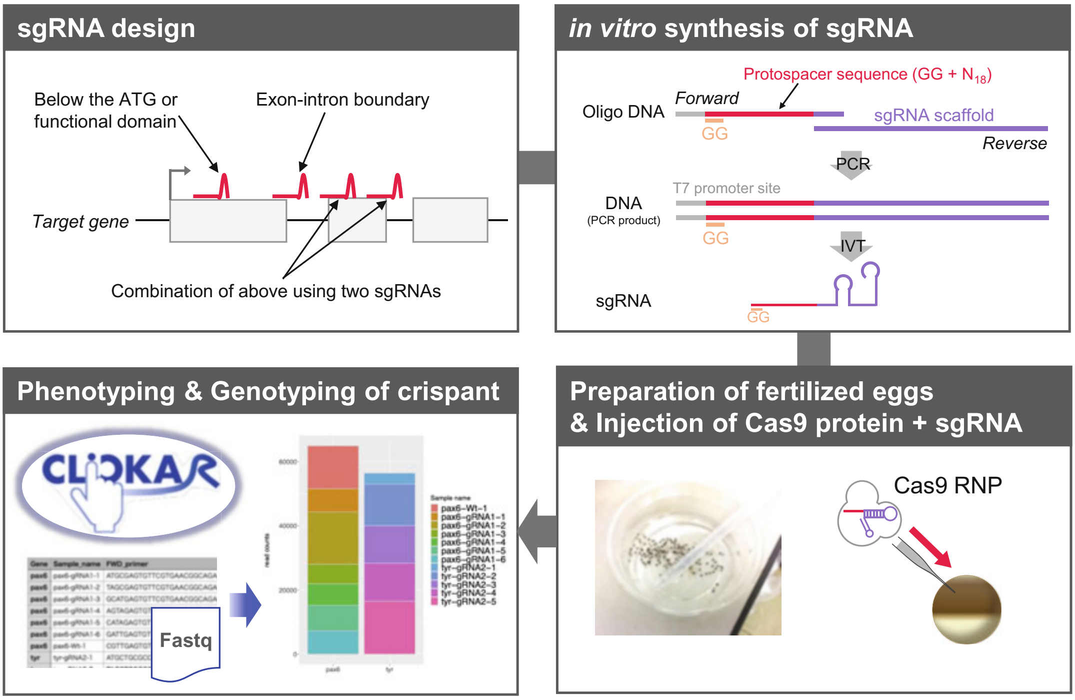

Fig. 1 A workflow for the crispant assay. Using Cas9 recombinant protein and commercially ordered oligonucleotides for the template DNA of sgRNA synthesis, crispants will be obtained within a week from the design of sgRNAs, without cloning

the sgRNA scaffold sequence and can be used in all PCR assemblies. *See* **Note 1**.

3. High-fidelity PCR enzyme (e.g., KOD FX Neo, TOYOBO).
4. Silica column-based PCR product purification kit (e.g., QIAquick PCR Purification Kit, Qiagen: 28104).
5. In vitro transcription kit based on T7 RNA polymerase (e.g., CUGA 7 gRNA Synthesis Kit, Nippon Gene: 314-08691).
6. Genome and transcript sequence(Frog, Xenbase(https://www.xenbase.org/); Newt, iNewt (http://www.nibb.ac.jp/imori/main/)).
7. Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.2 Preparation of In Vitro Fertilized Eggs

1. 10 × Marc's Modified Ringers (MMR) stock solution: 1 M NaCl, 20 mM KCl, 10 mM $MgSO_4$, 20 mM $CaCl_2$, and 50 mM HEPES; adjust pH to 7.4. Sterilize with autoclave or 0.2 μm filter. Store at room temperature for up to 1 year (Other ringers can be used. *See* **Note 3**).
2. 0.1 × MMR with 10 U/mL penicillin-streptomycin (e.g., add 1/1000 of 10,000 U/mL penicillin-streptomycin, Gibco). Store at room temperature for up to 1 month.

3. 2% (w/v) L-cysteine (in 0.1 × MMR), adjust pH to 7.8 with 10 N NaOH for frog. Use 0.5% L-cysteine without NaOH for newt. Prepare just before use.
4. 100 U/100 μL human chorionic gonadotropin (hCG). Dissolve in 0.6% NaCl solution and store at −20 °C. For *X. tropicalis*, 15 U/100 μL hCG is also needed.
5. 1% (w/v) MS-222.
6. 70% Leibovitz L15 medium diluted with sterilized water. Sterilize with a 0.2 μm filter. Store at 4 °C for up to 1 month. This only for *X. tropicalis.*
7. 1 × Modified Barth's Saline (MBS): 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 2.5 mM $NaHCO_3$, 1 mM $CaCl_2$, and adjust pH to 7.8. Sterilize with a 0.2 μm filter or autoclave. This is only for *X. tropicalis.*
8. Plastic pipette. Cut the tip to ensure size is large enough for egg collection.

2.3 Injection of Cas9 RNP

1. Microinjector (e.g., Nanoject II, Drummond Scientific).
2. Micromanipulator (e.g., Micromanipulator MM33, Drummond Scientific).
3. Stereomicroscope.
4. Incubator (8–26 °C).
5. Microneedle (e.g., 3.5 inch glass capillaries for Nanoject II, Drummond Scientific: 3-000-203-G/X).
6. Puller for microneedles (e.g., PN-31, NARISHIGE). *See* **Note 4**.
7. Mineral oil (e.g., Sigma: M8410).
8. 100 mm petri dish coated with 1% agarose dissolved in 0.3 × MMR. Store at 4 °C for up to 1 month.
9. 60 mm petri dish coated with 1.5% agarose dissolved in 0.3 × MMR. Store at 4 °C for up to 1 month. This is only for *X. tropicalis.*
10. Incubation buffer for injection: 5% (w/v) Ficoll PM400 (Sigma: F4375) in 0.3 × MMR with 10 U/mL penicillin-streptomycin. Sterilize with 0.2 μm filter (do not autoclave). Store at 4 °C for up to 3 months.
11. 10 μg/μL Recombinant Cas9 protein (Alt-R S.p. Cas9 Nuclease 3NLS, IDT: 1081058). *See* **Note 5**.
12. 10 × Cas9 buffer stock solution: 1.5 M KCl and 0.2 M HEPES (RNase-free).

13. Nuclease-free water (e.g., Thermo Fisher Scientific: AM9938). Do not use DEPC-treated water.

2.4 Genotyping

1. Genomic DNA extraction kit (e.g., DNeasy Blood and Tissue Kit, Qiagen: 69504).
2. High-efficient PCR enzymes suitable for genomic PCR (e.g., KOD FX Neo, Toyobo; KAPA HiFi HS Ready Mix, NIPPON Genetics).
3. Electrophoresis equipment. Agarose gel electrophoresis is fine for routine work, but a microcapillary electrophoresis system is better (e.g., Tape Station, Agilent; Multina, Shimadzu).
4. Locus-specific primer for heteroduplex mobility assay.
5. Locus-specific primer sets containing custom barcodes and overhang adaptor sequences of 16S Metagenomic Sequencing Library for amplicon-sequencing (Underlines, Illumina, Nextera adaptor sequence).

   Forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – custom barcode – target locus -3′). Reverse (5′--GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA – custom barcode - target locus -3′).
6. PCR product purification kit (e.g., QIAquick PCR Purification Kit, Qiagen: 28104).
7. A web tool for pooled amplicon sequence analysis, CLiCKAR (http://clickar.nibb.ac.jp/).

3 Methods

3.1 Design and In Vitro Synthesis of sgRNA

1. Find 5′-GG(N)$_{18}$-NGG-3′ or 5′-(N)$_{20}$-NGG-3′ sequence of the gene of interest (GOI). When we synthesize sgRNA with T7 RNA polymerase, GG comes to the 5′ end, but from our experience, it works well even if both GG mismatch to the genome. Therefore, you can simply find 5′-(N)$_{20}$-NGG-3′. *See* **Notes 2** and **6**.

   For species with a well-annotated genome (*X. laevis* and *X. tropicalis*), there are web-based sgRNA designing tools (e.g., *see* **Note** 7). For *P. waltl*, find the transcript sequence of the GOI from iNewt [27], check exon-intron structure by comparing to other species with blastx and Clustal Omega [28], and design sgRNAs on the coding region. The following sites are potentially good to induce a null knockout:

   - Just below the ATG sequence for a frameshift mutation
   - Functionally important domains

- Exon-intron boundary to disrupt pre-mRNA splicing
- Combination of the above using two or three sgRNAs

Multiple genes can be targeted by injecting two or more sgRNAs [1, 13, 15]. For allotetraploid *X. laevis*, targeting conserved sequences between both L and S homeologs can efficiently induce knockout in all four copies of genes [29]. You can also perform functional analysis of regulatory sequences by targeting the transcription factor binding domain [15]. To avoid misunderstanding the phenotypes from the off-target effect, we usually take the following precautions (*see* **Note 8** also):

- Design multiple sgRNAs to the same gene and confirm their phenocopy.
- Use a negative control sgRNA which has a 3–5 bp mismatch at the seed region of the protospacer (12 bp adjacent to the PAM, critical for the target specificity [30, 31]).

2. Copy and paste $(N)_{18}$ sequence of your target site into the forward oligonucleotide sequence in 2.1-1, and order it. *See* **Note 9**.
3. Prepare 80 μL of PCR reaction mix with 150 pmol of each forward and reverse oligonucleotide. PCR conditions are shown in Table 1.

Table 1
PCR conditions for sgRNA template assembly

Reagents	Volume (μl)
2 × PCR buffer for KOD FX Neo	40
2 mM dNTPs	16
100 pmol/μl forward primer	1.5
100 pmol/μl reverse primer	1.5
KOD FX Neo (1U/μl)	1.2
Nuclease free water	Up to 80 μl

The PCR cycle condition

94°C for 5 min
↓
94°C for 20 sec ←
60°C for 30 sec 10 cycles
68°C for 15 sec
↓
68°C for 1 min

4. Purify the PCR product with a column. Elute PCR product with 30–50 μL nuclease-free water. Check specific amplification by agarose electrophoresis, and measure concentration. PCR product size should be 117 bp.
5. In vitro transcribe and purify using CUGA 7 gRNA Synthesis Kit, following the manufacturer's guide. Add 150–200 ng of the purified PCR product as template DNA in a 20 μL reaction. Incubate at 37 °C for 2.5 h. Make sure to add DNase after transcription to remove the template DNA. Elute sgRNA with 30–50 μL of nuclease-free water. *See* **Notes 10–12**.
6. Quantitate the concentration. Check its quality by agarose electrophoresis. We usually obtain 50–100 μg. Dispense into nuclease-free tubes, and store at −80 °C. *See* **Note 13**. If the yield is low, *see* **Note 14**.

3.2 Preparation of Cas9 RNP

1. Dissolve Cas9 protein to 1 μg/μL with 1 × Cas9 buffer just before use.
2. Add 0.46 μL 10 × Cas9 buffer, 1 μL dissolved Cas9 protein, and 200 ng sgRNA, and adjust to 4.6 μL with nuclease-free water. The final concentration per egg (4.6 nL) is 1 ng Cas9, 200 pg sgRNA, 150 mM KCL, and 20 mM HEPES. For *X. tropicalis*, adjust 2.3 nL/egg with the same concentration. You can also use 2 ng Cas9 and 400 pg sgRNAs (or 200 pg of two different sgRNAs) per egg, maximum.
3. Incubate the mixture at room temperature for 15 min. Prepare just before use; keep on ice until use. *See* **Note 15**.

3.3 Preparation of In Vitro Fertilized Eggs

Good quality eggs are most important for the success of gene knockout. For details on handling each species, refer to McNamara et al. (2018) for *X. laevis* [32], del Viso and Khokha (2012) for *X. tropicalis* [33], and Hayashi et al. (2019) for *P. waltl* [34]. Every buffer and agarose plate should be adjusted to the incubating temperature a day before the experiment. The animal experiments should be approved and be performed according to animal ethics guidelines in your institution:

1. Inject hCG at the following amounts and timings:

   X. laevis: 450 U to females and 100 U to males 20 h before the fertilization

   X. tropicalis: 15 U to males 3–7 days before, 15 U to females and males 20 h before, and 100 U to females on the morning of the day of fertilization for the boost. Females start to lay after 3–4 h from the boost

   P. waltl: 100 U to females around 16 h before fertilization

2. Sperm preparation:

   X. laevis: Isolate their testes from deeply anesthetized two males and keep them in a 1.5 mL tube on ice. At the onset of egg collection, add 1 mL of 1 × MMR with one side of the testis from two males, and gently grind with pestles.

   X. tropicalis: Isolate their testes from deeply anesthetized males. Store testes in 70% Leibovitz L15 medium at 14–25 °C. At the onset of egg collection, remove L15, add 500 μL of 1 × MBS, and gently grind with pestles. *See* **Note 16**.

   P. waltl: Gently squeeze sperm from males; collect into 500 μL of 0.1 × MMR. Do not pipette. Sperm can be kept for a day at room temperature or 1 day at 4 °C.

3. Egg collection. Gently squeeze eggs from the females. Add sperm solution and mix gently. Leave them for 5 min at room temperature. For *X. tropicalis*, use an agarose gel-coated dish to prevent eggs from sticking on the dish.
4. Soak the dish with 0.1 × MMR. Keep at least 15 min.
5. De-jelly the embryos with L-cysteine. Proceed to wash within 3 min for *Xenopus*, 30 s for *P. waltl.*
6. Rinse the embryos with 0.1 × MMR several times. For *P. waltl*, remove jelly by two forceps manually after the rinse. After de-jellying, transfer embryos to 5% Ficoll in 0.3 × MMR.
7. Keep embryos at a cool temperature to delay the first cleavage (*X. laevis*: 18 °C, *X. tropicalis*: 22 °C, *P. waltl*: 8 °C). To reduce mosaicism, you have to inject Cas9 RNP before the first cleavage and as soon as possible after fertilization. *See* **Note 17**.

3.4 Injection of Cas9 RNP

1. Make 3–6 lines of V-shaped grooves on an agarose plate using a blade and align embryos (fertilized eggs) on it: 100 mm dish for *X. laevis* and *P. waltl* and 60 mm for *X. tropicalis.*
2. Set up microinjector, fill Cas9 RNP into the microneedles, and inject. The injection volume is 4.6 nL for *X. laevis* and *P. waltl* and 2.3 nL for *X. tropicalis*. For both *Xenopus*, finish injection within 30–40 min after de-jellying (by the first cleavage) to reduce mosaicism.
3. Incubate embryos under the following conditions: *X. laevis*, 18 °C; *X. tropicalis*, 26 °C; and *P. waltl*, Turn off the incubator to let them get to room temperature (~26 °C).
4. Selection of injected embryos: Transfer the embryos to 0.1 × MMR at the morula or blastula stage, because Ficoll disturbs gastrulation. Using a fresh plate and rinsing multiple times is also recommended. Select normally developing embryos. Timing is 3–4 h postinjection for *Xenopus* and next morning to noon for *P. waltl*. Following blastula stage, a non-agarose-coated dish can be used for *X. tropicalis.*

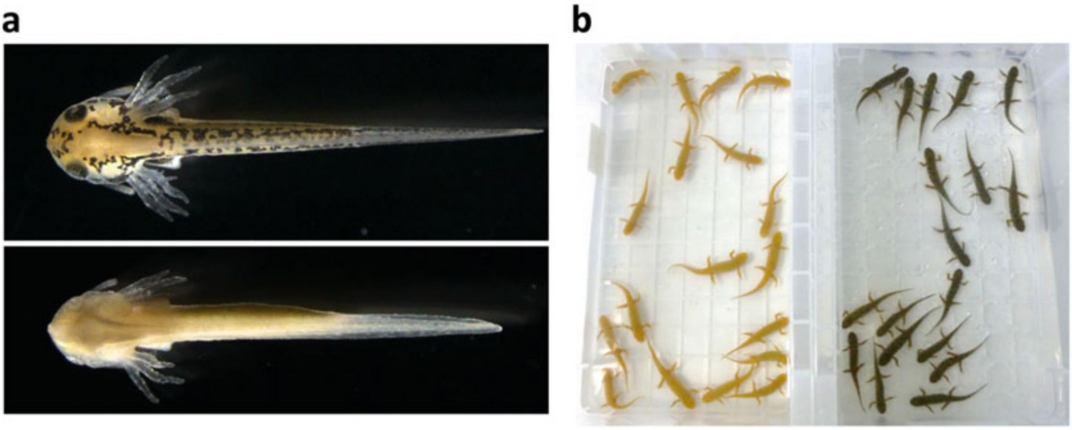

Fig. 2 Representative phenotypes of crispants. (**a**) An example of a *P. waltl* crispant. Wild-type (upper) and *Tyr* crispant (lower). Complete loss of pigmentation was observed in the founder generation. (**b**) Siblings from a single experiment. Uninjected control (right) and *Tyr* crispant (left)

5. Incubate embryos in the following conditions until the checkpoint of your desired phenotype: Change medium and remove abnormal embryos every day. *X. laevis*, 20 °C; *X. tropicalis*, 26 °C; *P. waltl*, room temperature (~26 °C). Representative phenotypes of crispants in *P. waltl* are shown in Fig. 2. For *X. laevis* and *X. tropicalis* crispants, refer to Shibata et al. (2022) and Shigeta et al. (2016), respectively [25, 29].

3.5 Genotyping Using the Heteroduplex Mobility Assay

In the crispant assay, it is important to confirm that the genotype is certainly correlated to the phenotype when you evaluate it. We introduce two different genotyping methods suitable for the crispant assay. One is the heteroduplex mobility assay (HMA) as a conventional method for routine work [1, 25], and the other is multiplexed amplicon-sequencing analysis for accurate calculation of somatic mutation and frameshift rate [1, 15, 25, 26, 35]. We usually set the amplicon size to 150–300 bp which can be used for both HMA and amplicon sequencing analysis.

When you perform genomic PCR on the target site with the crispant genome DNA, the wild-type and mutated alleles will be amplified simultaneously, and they form a heteroduplex if mutated sufficiently. A heteroduplex has different mobility to a homoduplex; therefore, you can easily confirm mutation by agarose gel or microcapillary electrophoresis of the PCR product. To facilitate heteroduplex annealing, make sure to set the cycle to more than 30 cycles (35 is recommended). You can use HMA to check mutation not only on-target sites but also off-target sites [25]. *See* **Note 18**:

1. Rear crispant to the stage you want to evaluate the phenotype and collect samples individually.

Table 2
PCR conditions for the heteroduplex mobility assay

Reagents	Volume (μl)
2 × PCR buffer for KOD FX Neo	5
2 mM dNTPs	2
10 pmol/μl forward primer	0.1
10 pmol/μl reverse primer	0.1
KOD FX Neo (1 U/μl)	0.15
Genomic DNA	10–100 ng
Nuclease free water	Up to 10 μl

The PCR cycle condition

94°C for 2 min
↓
98°C for 10 sec ← ⌉ 35 cycles
68°C for 30 sec ⌋
↓
68°C for 1 min

2. Extract genomic DNA from each sample using a silica column-based DNA purification kit. SDS/proteinase K treatment can be accepted, but make sure to perform phenol-chloroform extraction and ethanol precipitation carefully. Poor purification may cause trouble with genomic PCR. *See* **Note 19**.
3. Perform genomic PCR to produce 100–300 bp amplicons containing the sgRNA target site. The typical PCR conditions are shown in Table 2. *See* **Note 20**.
4. Electrophorese the PCR product. Figure 3 shows an example of an HMA assay of the microcapillary system. The PCR product from the uninjected (control) embryo became a single band (homoduplex of the wild-type allele), whereas up-shifted bands (heteroduplex of the wild and mutated alleles) were detected only in crispants. Polyacrylamide gel is also recommended. Conventionally, you can use agarose gel. *See* **Note 21**.

3.6 Genotyping Using Multiplexed Amplicon Sequencing

Amplicon sequencing analysis using NGS can accurately identify somatic mutation and frameshift rate in crispants and allows massively parallel analysis of multiple targets and individual crispants in a single sequencing run. Because sequencing cost has become increasingly cheap, amplicon sequencing is now a popular method for the genotyping of crispants in various organisms instead of sanger sequencing [15, 25, 35–39]. We usually amplify the target site with an overhang Nextera adaptor sequence of the 16S Metagenomic Sequencing Library Preparation Kit and send it to the

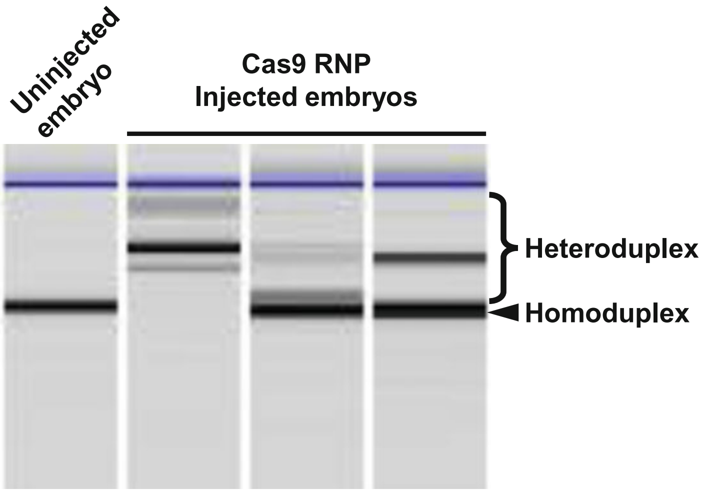

Fig. 3 Detection of CRISPR-Cas-mediated mutagenesis using the heteroduplex mobility assay. The PCR products containing the sgRNA target site from the uninjected control and crispants were electroporated using a microtip. The single band from the uninjected embryo is a homoduplex of the wild-type allele (closed arrowhead). Upshifted bands are heteroduplexes of the wild-type and mutant alleles (brackets) and were only detected in crispants

Miseq shared run services. There are various providers for Miseq shared run, and it costs around $200/50–100 K reads/sample. Sequence data can be easily analyzed through several web-based tools [40–46]. Graphical reports of mutation rates are obtained simply by uploading your raw fastq file. To genotype a large number of crispants, we add a custom barcode sequence to the primers so that the amplicons from 10 to 20 individuals could be pooled into one sequencing sample. To demultiplex and evaluate somatic mutations in each phenotype from the pooled sequence data, we developed an R program and a web-based tool, designated CLiCKAR [46]:

1. Design the locus-specific primers. Add overhang Nextera adaptor sequences of the 16S Metagenomic Sequencing Library (33 bp for both forward and reverse) and a 3–4 bp custom barcode sequence for recognizing each individual. Barcode sequences could be freely set by users. Order the primers for each individual. The amplicon size without the overhang sequence will be 150–550 bp, depending on the Miseq platform. For example, if you use a pair-end 300 bp, 550 bp [300 + 300 − 50 (to merge the pair-end reads) bp] will be the maximum size of the amplicon.
2. Perform genomic PCR. Recommended cycle number is around 28–30. It should not reach a plateau to refer to the original wild and mutated allele ratio to avoid the formation of heteroduplex amplicons.

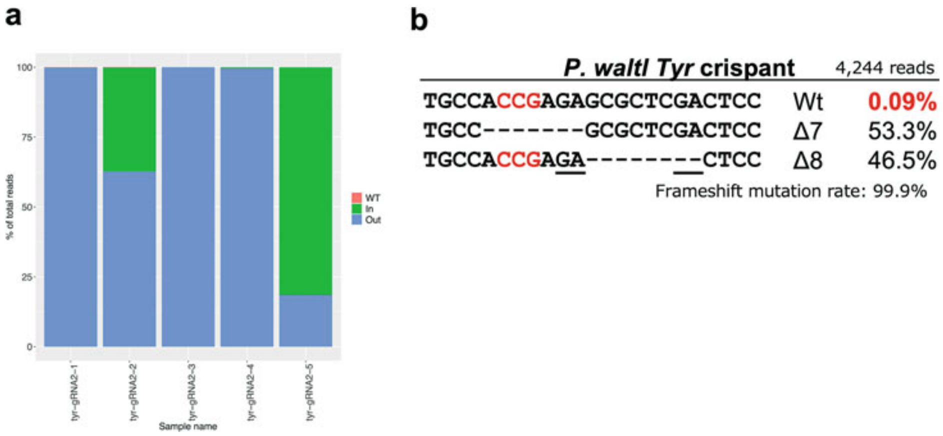

Fig. 4 Genotyping of crispant using amplicon-sequencing analysis. (**a**) A bar graph of mutation rate generated by CLiCKAR. Blue and green show frameshift and in-frame mutation rates of *Tyr* crispant of *P. waltl*, respectively. (**b**) Representative mutant alleles, their occupancy rates, frameshift mutation rate, and total read count are shown corresponding to the phenotype in Fig. 3a, calculated by CLiCKAR. Deletions are indicated by dashes. PAM and microhomologous sequences are indicated by red letters and underscore, respectively

3. Purify the PCR product with a column. Elute PCR product with 30–50 μL of nuclease-free water and measure concentration.
4. Mix equimolar amounts of the amplicons from ~10 individuals into a single tube. *See* **Note 22**.
5. Send to the NGS service provider in the Miseq platform.
6. Get fastq, and access CLiCKAR. Prepare a query table (csv file) that contains the IDs of each individual and the primer sequence with your custom barcode. Upload fastq and csv, and then follow the instructions. CLiCKAR gives you a graphical report of somatic and frameshift mutation rates and the list of each mutant allele and its counts for each crispant. Representative data are shown in Fig. 4. Cas9 cleaves 3 bp upstream from PAM; consequently, the indels are introduced there through the NHEJ or MMEJ repair. *See* **Note 23**.

4 Notes

1. The forward oligonucleotide contains a T7 promoter and protospacer targeting the GOI and a region of crRNA sequence. The reverse oligonucleotide contains a region of crRNA, a linker loop, and tracrRNA sequences (called sgRNA scaffold sequences) and is common to all sgRNA templates. The

scaffold sequence from the pDR274 plasmid has been described previously [47]. Because the 3′ terminal regions are partially complementary in each oligonucleotide, the DNA template for sgRNA can be produced by annealing and extension in the PCR assembly step.

2. To increase the in vitro transcript yield with T7 RNA polymerase, the +1 and +2 nucleotides from the transcription start site must be guanines (G); consequently, the 5′ terminal of the transcribed sgRNA becomes GG.
3. MMR can be shared in three species (*X. laevis*, *X. tropicalis*, and *P. waltl*). If you are already working on microinjection of amphibians, other buffers on your lab protocol can also be used (e.g., 10% Holtfreter's solution instead of 0.1 × MMR).
4. Settings vary for the puller, individually. Break the tip of the glass microneedle carefully under the microscope using fine forceps or a blade before use.
5. We have confirmed that the recent version (Alt-R® S.p. HiFi Cas9 Nuclease V3) also works with high efficiency as well as V2 in *Pleurodeles waltl*.
6. If you want to avoid GG mismatch, ordering crRNA and tracrRNA instead of in vitro synthesis also becomes another option [15].
7. Examples of online tools for sgRNA design, CRISPRdirect [48], Cas-Designer [49], CRISPRscan [50], Cas-OFFinder [51], and CHOPCHOP [52], can search the on- and off-target sequences on both the *Xenopus* genomes.
8. Injecting sgRNAs solely is toxic to the embryo, so we do not recommend using it as a negative control.
9. We do not add any purification options when ordering oligonucleotides (e.g., PAGE or HPLC), but it may be required depending on the distributers.
10. Do not scale down reaction volume.
11. The remaining template DNA affects the viability of the embryo, so the DNase step is mandatory.
12. The remaining surfactant or EtOH will affect survival of the injected embryo. Make sure to remove buffers by repeating and/or extending centrifugation in the wash step.
13. Do not freeze-thaw dispensed RNA; it should be for single use.
14. If you get an extremely low yield on a specific sgRNA, it will also have low cleavage activity. There might be an RNA secondary structure or some difficult sequence for an extension by T7 RNA polymerase. We do not recommend using it; better to choose a different sequence. In other words, you can screen

un-efficient sgRNA during in vitro transcription, but you cannot know when you determine the target sites.

15. *Important*: Dissolved Cas9 protein and Cas9 RNP solution should be prepared just before use. You cannot stock or refreeze them as a working solution. It significantly decreases efficiency. Cas9 RNP can be used for a day if it is kept on ice or at 4 °C.
16. Isolated *X. tropicalis* testis cannot be stored at 4 °C. We recommend using them on the same day because sperm motility decreases gradually over time.
17. The first cleavage occurs under the following conditions: *X. laevis*, 1 h for 18 °C; *X. tropicalis*, 40–50 min for 22 °C; and *P. waltl*, 5–6 h for room temperature.
18. The lowest mutation rate detectable in HMA is approximately 0.5–2.5% in human cultured cells, mice, and *X. tropicalis* [25, 53].
19. We sometimes use an alternative protocol as follows, modified from Nakayama et al. [23]: The samples are lysed in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 0.5% Tween 20) with a final concentration of 200 μg/ml proteinase K. After incubation at 56 °C for more than 2 h, phenol/chloroform extraction and ethanol precipitation are performed with linear polyacrylamide and sodium acetate. Resuspend the genome DNA in 20–40 μL of TE buffer (pH 8.0). Add 0.5 μL of the DNA solution to a 10 μL PCR.
20. The PCR products for HMA should not exceed 300 bp. It makes it hard to distinguish between heteroduplex and homoduplex bands by agarose electrophoresis.
21. If you use agarose gel, doublet bands form just above the homoduplex (normal PCR product). Refer to Figs. S1–S2 in Shiegta et al. (2016) for the example image [25].
22. The number of individuals that can be pooled will vary on how many reads you want to analyze for each crispant (50–100 K reads will be shared by the pooled samples). We usually mix up to 20 individuals, not to go below 1000 reads per sample.
23. More than 90% of mutations are observed within ±11 bp of the DSB site [25]. Using our protocol, the somatic mutation rates approach around 100% suggesting that an almost complete knockout of *Xenopus* and *P. waltl* can be generated in the founder generation [1, 15, 25, 46].

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Genome Editing of Silkworms

Takuya Tsubota, Hiroki Sakai, and Hideki Sezutsu

Abstract

Silkworm is a lepidopteran insect that has been used as a model for a wide variety of biological studies. The microinjection technique is available, and it is possible to cause transgenesis as well as target gene disruption via the genome editing technique. TALEN-mediated knockout is especially effective in this species. We also succeeded in the precise and efficient integration of a donor vector using the precise integration into target chromosome (PITCh) method. Here we describe protocols for ZFN (zinc finger nuclease)-, TALEN (transcription activator-like effector nuclease)-, and CRISPR/Cas9-mediated genome editing as well as the PITCh technique in the silkworm. We consider that all of these techniques can contribute to the further promotion of various biological studies in the silkworm and other insect species.

Key words Silkworm, ZFN, TALEN, CRISPR/Cas9, Knock-in, PITCh

1 Introduction

Silkworm, *Bombyx mori*, is a lepidopteran insect that has been used as a model in various biological studies including physiology, biochemistry, pathology, and genetics. In 2000, the transgenic technique was established [1], and this greatly contributed to the facilitation of a wide variety of silkworm studies including gene functional analysis as well as practical research. The availability of the genome sequence [2] is also valuable not only for silkworm studies but also for other lepidopteran species. It is obvious that this is the most advanced model insect in this order.

The genome editing of the silkworm was firstly carried out using ZFN technology [3]. In this study, the knockout of the *biogenesis of lysosome-related organelles complex 1, subunit 2* (*BLOS2*) gene, was attempted, and this resulted in the successful disruption of this gene. *BLOS2* is an excellent model for genome editing assay for the following reasons; first, its mutant shows a translucent skin phenotype that can be easily detected [3, 4]

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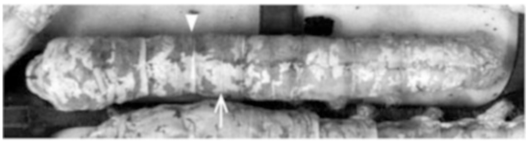

Fig. 1 Oily mosaic G0 larva caused by the *BLOS2* knockout. The arrow indicates the normal epidermis region and the arrowhead depicts the oily skin

(Fig. 1), and second, *BLOS2* is located on the Z chromosome that permits the germline knockout detection in the hemizygous females in G1.

After the success of the ZFN-mediated knockout, another programmable nuclease TALEN was developed, and the effectiveness of this tool was evaluated in the silkworm using *BLOS2* as a model. Initially the efficiency of TALEN was comparable to that of ZFN [5]; however, the TALEN vector was improved so that the synthesized RNA can be compatible with the silkworm translation machinery, or the TALEN activity can be expressed at a higher level. The utilization of this vector increased knockout efficiency by more than one order of magnitude [6]. Due to this result, a number of knockout studies are ongoing using this improved TALEN [7–11].

CRISPR/Cas9, another genome editing tool by RNA, has been adapted as an efficient and easy genome editing tool for many animals. Like other animals, CRISPR/Cas9-mediated knockout has been available for silkworm [12, 13]. Genome editing by CRISPR/Cas9 is a standard method to induce mutations and study how specific genes work in animals. Therefore, the way of CRISPR/Cas9-mediated knockout can be referred to the knowledge of many animals, and the labor of experiments can be greatly reduced by purchasing Cas9 protein or gRNA synthesis Kit. The CRISPR/Cas9-mediated knockout is functional in the silkworm, though its effectiveness is generally less than TALENs [14]. However, due to the simplicity of the experiment, knockout using CRISPR/Cas9 has been widely conducted in silkworm [12].

Targeted integration of the donor vector is another important application of genome editing. Conventionally this was approached via the homology-directed repair (HDR) pathway, but the efficiency was very low in the silkworm and other species presumably due to the variable frequencies of homologous recombination in cell types and organisms [14]. To overcome this, the donor vector was designed so that it can be linearized by TALENs, and the microinjection of this arranged vector in mixture with TALENs could successfully cause site-specific integration with comparable efficiency to transposon-mediated transgenesis in the silkworm [15]. In contrast to this method, we recently developed another knock-in system termed PITCh [16]. This method is based on a microhomology-mediated end-joining (MMEJ) pathway, a recently identified DSB repair mechanism that uses microhomologous sequences (5–25 bp) for error-prone end-joining [17]. We demonstrated that the TALEN-based PITCh system (TAL-PITCh) could efficiently integrate a donor vector harboring the *hsp90* promoter and GFP into the *BLOS2* genomic locus in a very precise manner [16]. The PITCh system was also effective in mammalian cell culture and in frogs [16, 18]. It is possible that this technique can be utilized in a wide variety of organisms.

Here, we describe protocols of ZFN-, TALEN-, and CRISPR/Cas9-mediated genome editing in the silkworm. In addition, we also describe the method of the TAL-PITCh system using *BLOS2* as a model. We consider that all of these techniques will be promising tools for the further promotion of silkworm and other insect studies.

2 Materials

2.1 ZFN-Mediated Knockout

1. Silkworms and Related Resources.

   Silkworms are available from the public bioresource center in Japan (National BioResource Project, http://silkworm.nbrp.jp/index_en.html; Genebank Project NARO, https://www.gene.affrc.go.jp/about_en.php), India (India Central Sericultural Germplasm Resources Centre http://csgrc.res.in/indent-germplasm.htm), and other countries. The silkworm gene information is available in KAIKOBASE (https://kaikobase.dna.affrc.go.jp) [19], SilkBase (https://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) [20], and SilkDB (https://silkdb.bioinfotoolkits.net) [21]. Silkworms can be reared using an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) or mulberry leaf. We usually rear on the artificial diet because we need to constantly have nondiapausing strains for the microinjection experiments (see below).
2. Equipment required for the molecular biological experiments.
3. Genome DNA extraction kit such as DNeasy Blood and Tissue Kit (QIAGEN).
4. PCR enzymes.
5. Sequencing reagents.
6. Standard entry vector such as pENTR-NLS-ZFN.
7. Clonase reaction destination vector such as pDEST14 with T7 sequence.
8. T4 DNA ligase.
9. *Escherichia coli* competent cells.
10. Plasmid midiprep kit.

11. Capped RNA transcription kit such as mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific).
12. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
13. A micromanipulator and microinjection system.

2.2 TALEN-Mediated Knockout

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. Genome DNA extraction kit such as DNeasy Blood and Tissue Kit (QIAGEN).
4. PCR enzymes.
5. Sequencing reagents.
6. TALEN construction tools such as plasmids from Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene).
7. Vector such as pBlue-TAL vector (Addgene).
8. *E. coli*-competent cells.
9. Plasmid midiprep kit.
10. Capped RNA transcription kit such as mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific).
11. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
12. A micromanipulator and microinjection system.

2.3 CRISPR/Cas9-Mediated Knockout

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. The PCR primers for a template-free PCR to produce double-stranded DNA template for T7 in vitro transcription [22, 23]. Here we utilize the following: CRISPR F contains the T7 promoter and the specific target sequence, 5′–GAATT AATACGACTCACTATA GGN$_{18-20}$ GTTTTAGAGCT AGAAATAGC–3′ (*see* Subheading 3.3, **step 2**), and CRISPR R primer can be used for all targets, 5′AAAAGCACCGACT CGGTGCCACTTTTTCAAGTTG ATAACGGACTAGCC TTATTTTAACTT GCTATTTCTAGCTCTAAAAC–3′.
4. PCR enzymes.
5. S.p. Cas9 nuclease such as Cas9 Nuclease V3 (Integrated DNA Technologies).
6. In vitro transcription kit such as Guide-it sgRNA In Vitro Transcription Kit (TAKARA).
7. A micromanipulator and microinjection system.

2.4 TAL-PITCh-Mediated Knock-in

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. The template plasmid for PITCh vector construction. Here we utilize pBachsp90GFP-3xP3DsRed, a plasmid with an *hsp90* promoter and GFP [16, 24] (*see* **Note 1**).
4. The PCR primers for inverse PCR from the template plasmid. Here we utilize: BLTS6DsRedF1 (5′-CCGATCCTAGCCGCGAACCTGAACAATAGTCACCACCTGTTCCTGTAG-3′) and BLTS6pBacLR1 (5′-CGGCTCCTTATACTTGGTGATCGCGCTCGAATTAGATCTTTGG-3′) (see below).
5. PCR enzymes.
6. T4 DNA ligase and kinase.
7. *E. coli*-competent cells.
8. Plasmid miniprep kit.
9. Plasmid midiprep kit.
10. Phenol/chloroform.
11. 100% ethanol.
12. 70% ethanol.
13. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0) and 5 mM KCl.
14. A micromanipulator and microinjection system.
15. A stereo fluorescence microscope.
16. PCR primers for detection of the knock-in. BLOS2F1 (5′-AGCAAATTGTTGCAAGGGAC-3′) and g2900_120R (5′-ATTTGTTGGCAGCACTGCTT-3′) are utilized here for the detection of the 5′ junction, and pBacL3490 (5′-ATAACGACCGCGTGAGTCAA -3′) and BLOS2R1 (5′-AGCTAGCCACAGTGTTTATC-3′) are used for the 3′ junction.
17. Agarose.

3 Methods

The outline of the knockout experiment (Subheadings 3.1, 3.2, and 3.3) is shown in Fig. 2.

3.1 ZFN-Mediated Knockout

1. Select the target site using the "Zinc Finger Tools" program (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php) [25].
2. Extract the genome from the *w1 pnd* strain (*see* **Note 2**) using a genome extraction kit. Amplify the target genome region by PCR. Analyze its sequence and determine the presence or absence of the polymorphic nucleotide in the ZFN target site.

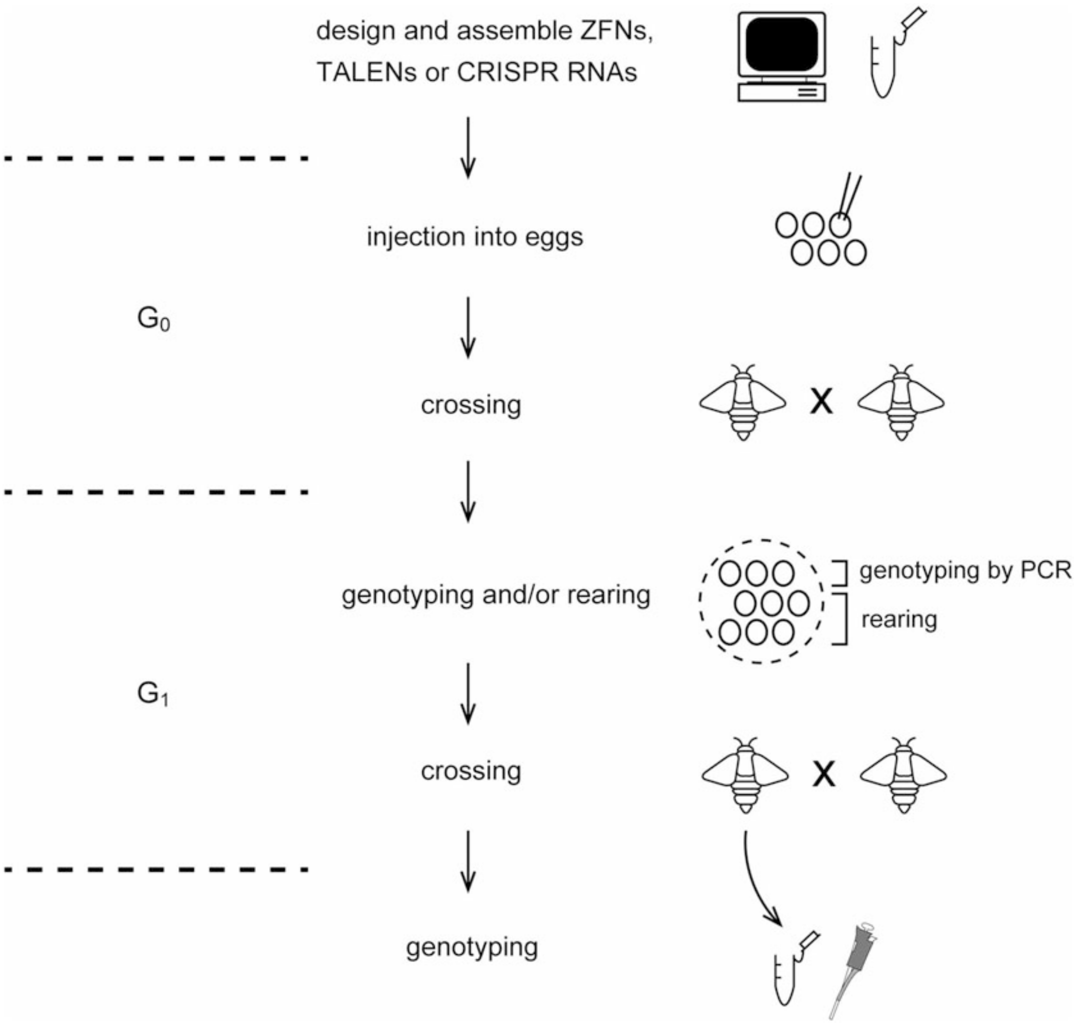

Fig. 2 Diagram of the genome editing experiment for the generation of *B. mori* knockout individuals

3. Construct the ZFN expression vector following the method described previously [3, 26].
4. Carry out the in vitro transcription using a kit such as mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific). Dissolve 100 ng/μl of each RNA in injection buffer.
5. Collect eggs from the *w1 pnd* strain by mating newly enclosed adult moths.
6. Inject the ZFN RNA using a microinjector (*see* **Note 3**). Seal the eggs using bonds.
7. Incubate the eggs keeping the humidity until hatched.
8. Rear the G0 individuals until adulthood and obtain fertile eggs by sib-mating or mating with non-injected individuals.

9. Incubate the eggs at 25 °C and collect 25–50 eggs from each brood for genomic DNA extraction.
10. Amplify the target region by PCR. Analyze the sequence of the PCR product and examine whether the mutation is present. For broods in which the mutation could be detected, rear the remaining individuals.
11. Cross the G1 adults with *w-c* or other diapausing strains to keep stocks. After crossing, extract the genome from each G0 adult and check the genotype by PCR and sequencing.

3.2 TALEN-Mediated Knockout

The detailed protocol for a TALEN-mediated knockout was previously published [27, 28]:

1. Select the TALEN target site. In comparison to ZFN, the TALEN target selection is flexible, and the target site can be selected at almost any region in the gene of interest [27, 28]. It is preferable that a natural restriction enzyme site exists within the target.
2. Assemble TALEN using tools such as Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene). Insert the assembled arrays into a vector such as pBlue-TAL [6] which is an expression vector optimized for the silkworm translation system. Utilization of this vector is critical for a highly efficient knockout.
3. Carry out the in vitro transcription using a kit such as mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific). Dissolve 125 ng/μl of each RNA in injection buffer.
4. Microinject the RNA into the *w1 pnd* eggs, and generate the knockout strain following the method described in Subheading 3.1 (*see* **Note 4**).

3.3 CRISPR/Cas9-Mediated Knockout

Previously, the genome editing of silkworm using the CRISPR/Cas9 system injected Cas9 mRNA [29]. Recently, it has been reported that injection of Cas9 protein is more effective than those using Cas9 mRNA in *B. mori*, because the protein acts immediately after injection without translation delay [30, 31]. In this chapter, we introduce the method using Cas9 protein:

1. Select the target site using CRISPRdirect (https://crispr.dbcls.jp/) [32], CHOPCHOP (https://chopchop.cbu.uib.no) [33], or other target selection tools.
2. Design the unique oligonucleotides, CRISPR F (5′–GAAATTAATACGACTCA CTATA GGN$_{18–20}$ GTTTTAGAGCTAGAAATAGC–3′). CRISPR F contains the T7 polymerase binding site and the specific target sequence (N$_{18–20}$), which is not including the PAM sequence. The target sequence is ideally GGN$_{18}$, which includes the first two

obligate nucleotides transcribed by the T7 RNA polymerase. If the specific sequence does not start with GG, extend it to 21 (GN_{19}) or 22 (GGN_{20}) base pairs [23]. However, it was reported that sgRNAs with 5′ matched GG are not necessarily more efficient than sgRNAs with 5′ GG mismatches for targets within the same gene in *B. mori* [30].

3. Perform a template-free PCR to anneal in two overlapping oligonucleotides (CRISPR F and CRISPR R) and produce a full-length dsDNA template.
4. Carry out the in vitro transcription using a kit such as Guide-it sgRNA In Vitro Transcription Kit (TAKARA).
5. Mix the sgRNA and Cas9 protein at a concentration of 100 ng/μl of sgRNA and 500 ng/μl of Cas9 protein. In order to form the ribonucleoprotein (RNP) complex, a mixture of sgRNA and Cas9 protein was incubated at room temperature for 15 min.
6. Follow the protocol described in Subheading 3.1 (*see* **Note 5**).

3.4 Knock-in Using TAL-PITCh

In this chapter, we present the MMEJ-assisted knock-in using TAL-PITCh. The gene knock-in using CRISPR-Cas9 with the PITCh systems probably works in silkworm; however, the knock-in efficiency is not a practical stage for now. We are now trying to make a good method that is the knock-in using CRISPR-Cas9 systems for silkworm. Here we describe a protocol of integrating a plasmid with the *hsp90* promoter-GFP (*see* Subheading 2.3, **item 4**) into the *BLOS2* locus using TAL-PITCh. The flowchart of the knock-in experiment is shown in Fig. 3:

1. Select the TALEN target site as described in Subheading 3.2, **step 1**. Here we select exon 3 of the *BLOS2* gene as the target (Fig. 4).
2. Design the donor (PITCh) vector. In the TAL-PITCh system, the PITCh vector needs to be designed so that TALENs targeting the genomic site can also cleave this vector [16, 34]. The TALEN recognition site should therefore be introduced into the PITCh vector (Fig. 5). At this time, the anterior and posterior half of the spacer sequence should be switched compared to that of the genomic target [16, 34] (Fig. 4). Following these rules, we can design the TALEN target sequence in the vector (Fig. 4).
3. Construct the PITCh vector. Design primers including the vector sequence and TALEN target (BLTS6DsRedF1 and BLTS6pBacLR1, Fig. 6). Perform PCR from pBachsp90GFP-3xP3DsRed (*see* **Note 6**) and self-ligate the PCR product. Transform into *E. coli*-competent cells.

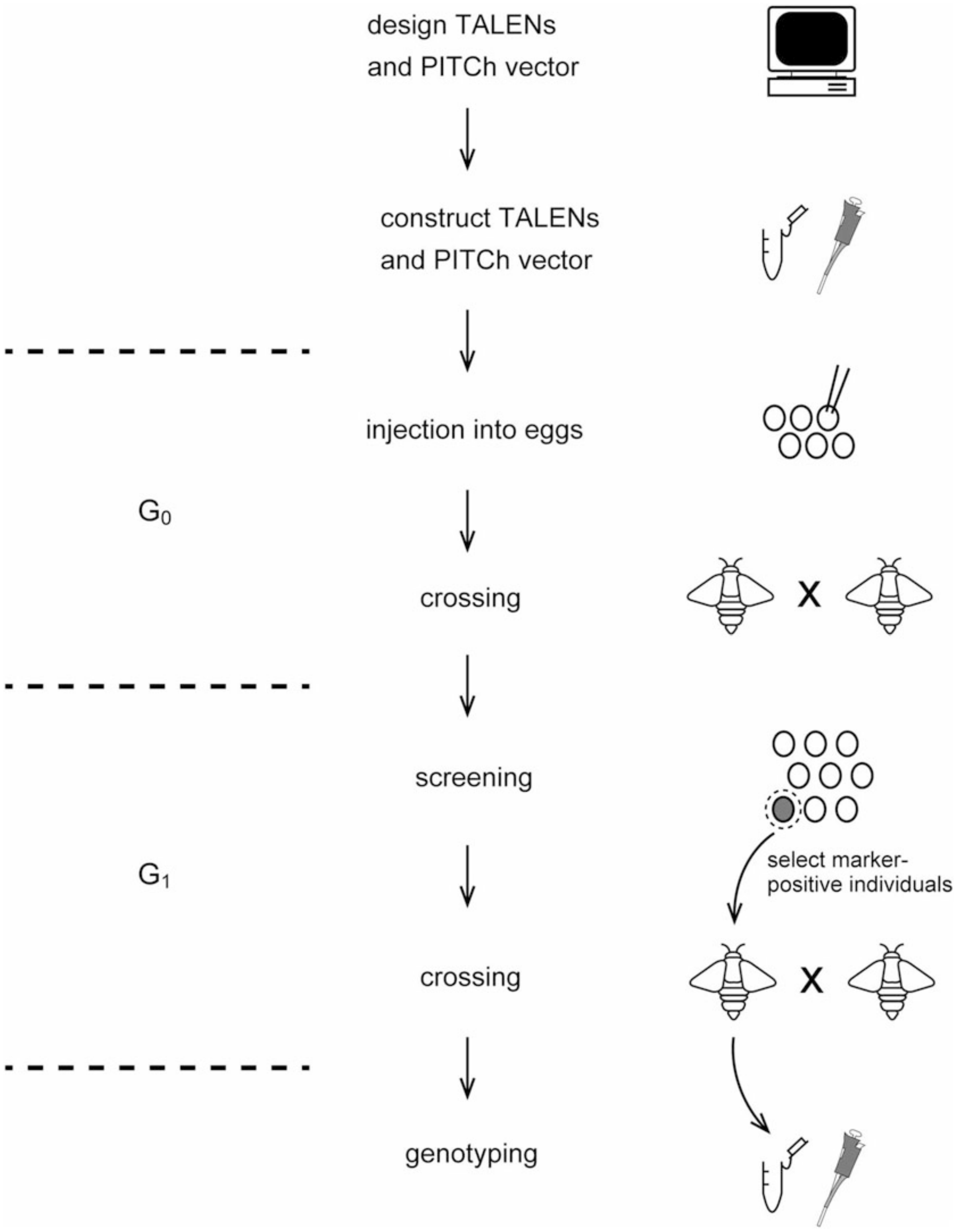

Fig. 3 Diagram of the genome editing experiment for the generation of *B. mori* knock-in individuals

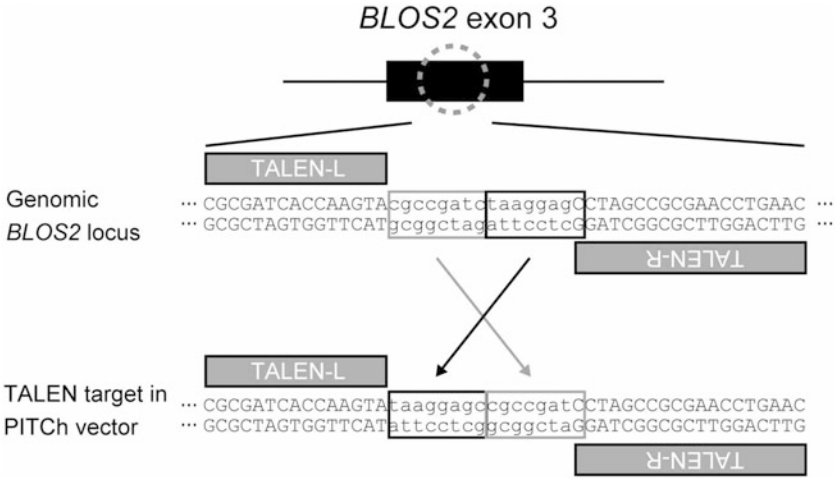
BLOS2 exon 3
TALEN-L
Genomic
BLOS2 locus
··· CGCGATCACCAAGTAcgccgatctaaggagCCTAGCCGCGAACCTGAAC ···
··· GCGCTAGTGGTTCATgcggctagattcctcGGATCGGCGCTTGGACTTG ···
TALEN-R
TALEN-L
TALEN target in
PITCh vector
··· CGCGATCACCAAGTAtaaggagccgccgatCCTAGCCGCGAACCTGAAC
··· GCGCTAGTGGTTCATattcctcggcggctaGGATCGGCGCTTGGACTTG
TALEN-R

Fig. 4 Design of the microhomology sequence

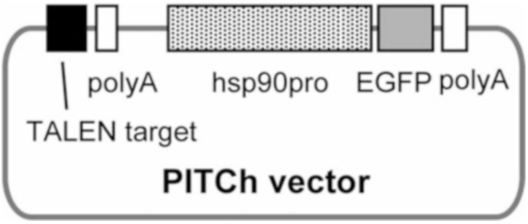

Fig. 5 Architecture of the PITCh vector

4. Pick 4–6 colonies. Purify the plasmid using a plasmid miniprep kit and verify the sequence around the TALEN target site. Occasionally, mutations can exist within the primer sequence utilized for the inverse PCR.
5. For colonies with the appropriate plasmid, purify again using a midiprep kit.
6. Extract the plasmid with phenol/chloroform, precipitate with 100% ethanol, wash with 70% ethanol three times, and dry. Suspend in injection buffer.
7. Mix TALENs and the PITCh vector at 125 ng/μl for each TALEN and 500 ng/μl for the PITCh vector.
8. Microinject the TALEN and PITCh vector into the *w1 pnd* eggs (*see* **Note** 7), and obtain the G1 broods following the method described in Subheading 3.1, **steps 6–8**.
9. Observe the fluorescence of the G1 embryos. According to the activity of the *hsp90* promoter, the GFP expression can be detected as early as 2–3 days after egg laying [24]. The GFP-positive individuals are possible candidates for knock-in (Fig. 7). Select the GFP-positive individuals and keep rearing (*see* **Note 8**).

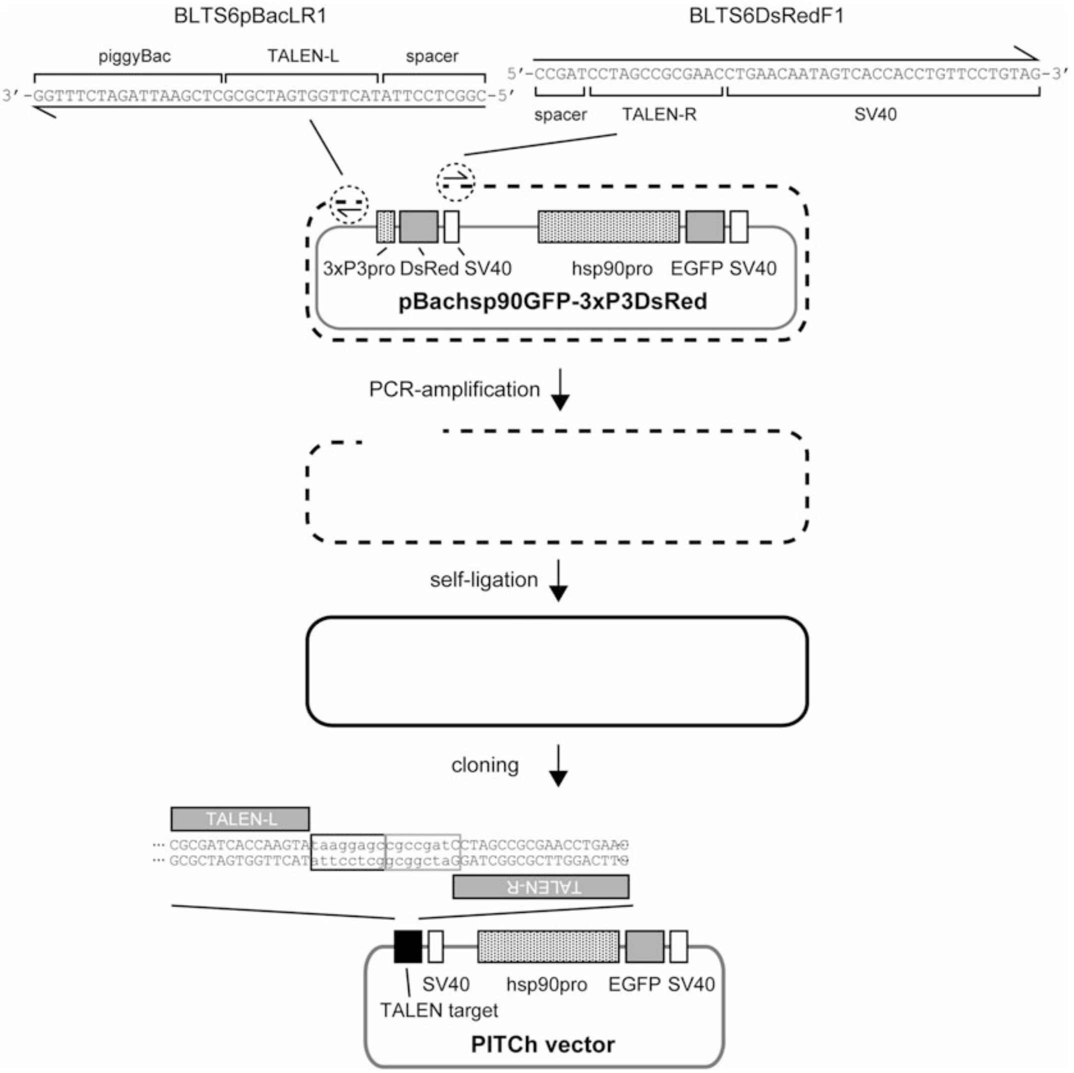

Fig. 6 Diagram of PITCh vector construction via inverse PCR. The TALEN target site can be inserted into the pBachsp90GFP-3xP3DsRed plasmid using primers with the shown sequences

10. Cross the GFP-positive individuals with a diapausing strain such as *w-c* and obtain fertile eggs. After crossing, extract the genome from each adult.
11. Perform PCR using primers designed against the vector and genome sequence (Fig. 8). Analyze the PCR product using gel electrophoresis (Fig. 9).
12. Verify the sequence of the PCR product and evaluate whether the knock-in has occurred as planned. In the case of *BLOS2*, knock-in into the targeted locus was detected for six individuals (4, 8, 16, 17, 34, and 35; Fig. 9) [16]. Among these individuals, precise knock-in occurred for both the 5′ and 3′

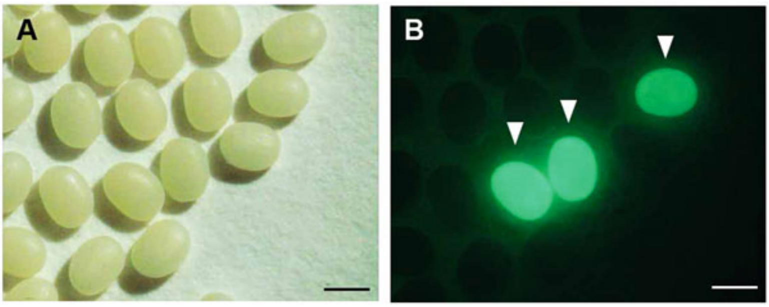

Fig. 7 Microscopy images of G1 embryos for the knock-in experiment. (**a**) Bright field. (**b**) Fluorescence. The arrowheads indicate GFP-positive individuals. Scale bar, 1 mm

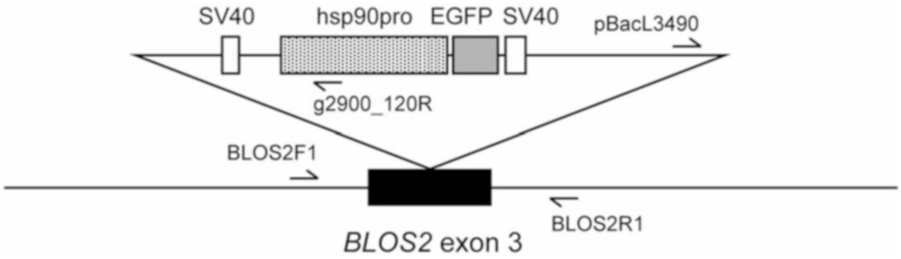

Fig. 8 Primer position for knock-in detection

junctions for 4, 8, 16, and 17, whereas for 34 and 35, insertion of extra nucleotides (around 1.7 kb) was present only in the 3′ junction (Fig. 9) [16].

4 Notes

1. The silkworm *hsp90* promoter exhibits strong and ubiquitous transcriptional activation activity in various developmental stages [24], and thus utilization of this promoter permits easy knock-in detection. Besides this promoter, the 3xP3 [35], *ie1* [36], *A3* [1] and *fibroin light chain* promoters [37] are available in the silkworm. These promoters are connected with marker genes such as the fluorescent protein genes EGFP, DsRed and CFP, or genes related to larval coloration such as kynurenine 3-mono oxygenase (KMO) [38] and arylalkylamine-*N*-acetyl transferase (aaNAT1) [39] for use as knock-in reporters.
2. We recommend the use of a nondiapausing strain for injection, although the silkworm genome sequence has been determined for the diapausing strain *daizo* [2]. The silkworm is a polymorphic organism, and it is critically important to determine the target site sequence in the strain actually being utilized for the

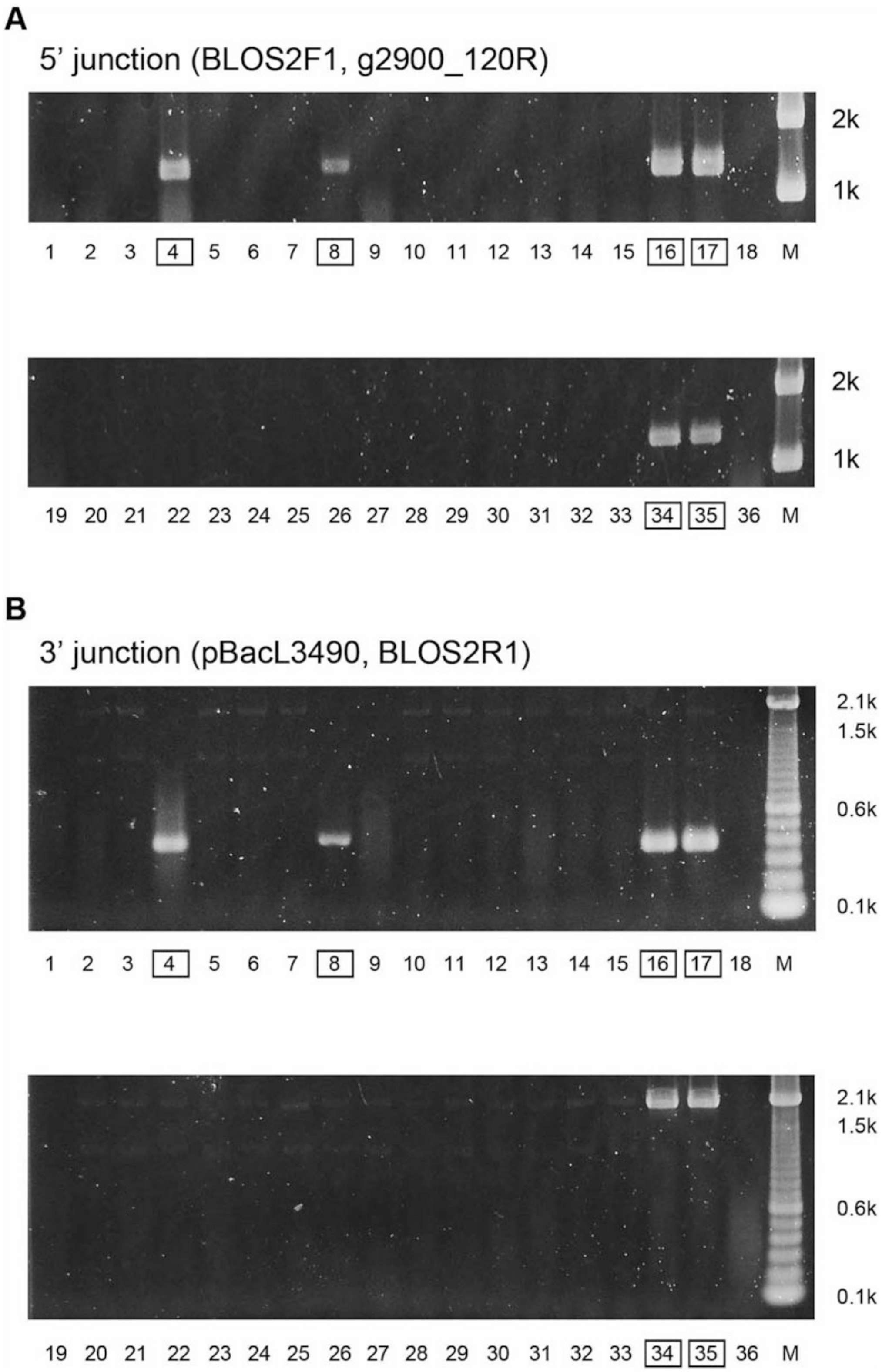
A
5' junction (BLOS2F1, g2900_120R)
2k
1k
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
2k
1k
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 M
B
3' junction (pBacL3490, BLOS2R1)
2.1k
1.5k
0.6k
0.1k
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
2.1k
1.5k
0.6k
0.1k
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 M

Fig. 9 Genotyping of *BLOS2* knock-in individuals. The PCR was carried out using the primers shown in Fig. 8, and the product was analyzed by gel electrophoresis. (**a**) is the 5′ junction and (**b**) is the 3′ junction. A total of 36 investigated individuals are shown here

microinjection (not only for the ZFN experiments but also for TALEN and CRISPR/Cas9). In our laboratory, we usually use *w1 pnd*, a nondiapausing strain with a white egg color mutation. This permits the easy detection of transgenic marker gene expression and is therefore suitable for the knock-in experiment.

3. We could obtain the *BLOS2* knockout strains from at least five broods by injecting 480 embryos [3].
4. TALEN efficiency is very high, and in most cases, injection into ~100 embryos is sufficient for knockout strain generation [6].
5. In the case of CRISPR/Cas9, microinjection into ~150 embryos is sufficient for knockout strain generation if the synthesized sgRNA is functional.
6. Preferably polymerases with high fidelity should be used.
7. We could obtain four individuals that showed precise integration for both the 5′ and 3′ junctions by injecting ~200 embryos [16].
8. All the GFP-positive individuals should be reared because in many cases the donor vector is not integrated into the targeted genomic locus [16].

Acknowledgments

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Improved Genome Editing in the Ascidian *Ciona* with CRISPR/Cas9 and TALEN

Yasunori Sasakura and Takeo Horie

Abstract

The ascidian *Ciona intestinalis* type A (or *Ciona robusta*) is an important organism for elucidating the mechanisms that make the chordate body plan. CRISPR/Cas9 and TAL effector nuclease (TALEN) are widely used to quickly address genetic functions in *Ciona*. Our previously reported method of CRISPR/Cas9-mediated mutagenesis in this animal has inferior mutation rates compared to those of TALENs. We here describe an updated way to effectively mutate genes with CRISPR/Cas9 in *Ciona*. Although the construction of TALENs is much more laborious than that of CRISPR/Cas9, this technique is useful for tissue-specific knockouts that are not easy even by the optimized CRISPR/Cas9 method.

Key words Ascidian, Tunicate, *Ciona*, Knockout, Germline mutagenesis

1 Introduction

Ascidians are a major group of the subphylum Tunicata of the phylum Chordata (*see* **Note 1**) [1]. Their phylogenetic position suggests that ascidians share a basic body plan with vertebrates. Indeed, ascidians possess chordate-specific characteristics such as the notochord, dorsal neural tube, pharyngeal gills, and endostyle/thyroid gland. The similarity between vertebrates and ascidians is best represented by the tadpole shape of ascidian larvae (Fig. 1). In addition, ascidians generally have very simple bodies compared to vertebrates. The tadpole larvae of ascidians consist of countable numbers of cells, and their cell lineages during embryogenesis have been described in detail [2]. These features enable us to observe the cellular and molecular mechanisms underlying the construction of chordate-specific structures [3].

Among ascidians, *Ciona intestinalis* type A (which was recently suggested to be renamed *Ciona robusta*; [4]) is a model ascidian species whose gene functions can be studies through genetic approaches. The genome sequence of *Ciona* has been well

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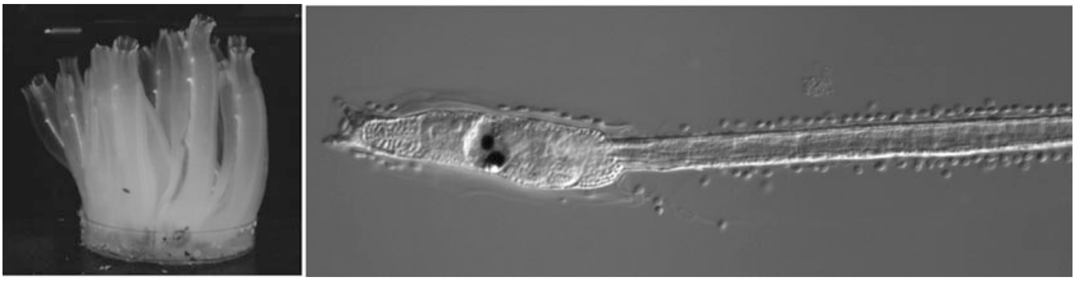

Fig. 1 The ascidian *Ciona intestinalis* type A. Left shows adults cultivated by the National BioResource Project Japan. Several animals are shown. The diameter of the petri dish at the bottom is 9 cm. Right shows a tadpole larva of *Ciona intestinalis*. The posterior portion of the tail is omitted from the panel. The body length of a larva is approximately 1 mm

annotated, enabling us to access information from genes quickly [5–7]. Methods to introduce exogenous nucleotides by microinjection or electroporation into eggs are routinely used to knock down or knock out genes by antisense nucleotides, dominant-negative forms of proteins, and genome editing tools [8, 9]. The *Ciona* genome is short and compact. A recent study estimated that the genome size of *Ciona* is 114–120 megabase pairs (kbp) per haploid, in which 14,000 genes are encoded [10]. This means that one gene is encoded in less than every 10 kbp of the genome. Reflecting the genome's high genetic density, the *cis* elements that control the spatiotemporal pattern of gene expression are short, allowing us to isolate tissue-specific enhancers easily. Indeed, many enhancers that are responsible for organ-specific, tissue-specific, or cell-type-specific expression have been isolated [9, 11]. The *cis* elements are used to express genetic tools for addressing gene functions in detail. *Ciona* has a relatively short generation time and can be cultured in laboratories [12], indicating that genetic approaches are feasible in *Ciona*. Indeed, transgenic and mutant lines of *Ciona* have been created by transposon-based transgenesis [13–15]. The availability of genetics for *Ciona* is exceptional in marine invertebrates, because inland culturing of marine invertebrates is usually difficult. These developmental and genetic techniques have advanced our understanding of the developmental mechanisms of *Ciona*.

In 2012, our group made the first report of genome editing in *Ciona*. In that report, we used zinc finger nuclease (ZFN) targeting *GFP* inserted in the genome [16]. In 2014, our group reported genome editing with transcription activator-like effector nuclease (TALEN) [17]. Construction of TALENs is simpler than that of ZFNs, particularly with the aid of the Platinum TALEN kit [18–20]. The TALENs constructed with this kit have mutation efficiencies sufficiently high to yield phenotypes reproducibly at G0 generation [17]. In the same year, we and another group reported

CRISPR/Cas9-mediated mutagenesis in *Ciona* [21, 22]. In these reports, guide RNAs (strictly, single-guide RNAs or sgRNAs) are supplied as in vitro synthesized RNAs or from expression vectors using polIII-dependent promoters. After the first two reports, a number of manuscripts using CRISPR/Cas9 in *Ciona* were published [23–32]. This genome editing system has been used not only in the simple knockout of protein coding genes but also in the knockin of a reporter gene tag in the target sites [25]. In a related ascidian, *Ciona savignyi*, CRISPR/Cas9 is used to monitor expression of small RNAs [33]. These applications suggest the great potential of the CRISPR/Cas9 system to improve genetic and genomic studies in *Ciona*.

Despite these splendid achievements, improvements in mutation efficacy by CRISPR/Cas9 have not necessarily been pursued well in *Ciona*. Because the rate of such efficacy is critical for determining the efficiency of experiments using this system, further progress in *Ciona* studies requires the validation and improvement of protocols. In our previous report [21], we used laboratory-made, in vitro synthesized guide RNAs or their expression vectors from an RNA polymerase III-dependent promoter [34]. Cas9 was also provided by synthesized mRNA or an expression vector using the *EF1α* promoter, which induces ubiquitous expression [35]. There were, however, two major limitations to the CRISPR/Cas9 system in *Ciona* compared to TALENs. First, a guide RNA has a low chance of yielding a detectable mutation rate. We made eight guide RNAs, and only two of them had mutation rates that could be detected by the Cel-I endonuclease-mediated method. Assuming that this low chance is generally applicable to guide RNAs constructed in the same way, we need to screen four sgRNAs to mutate one gene by CRISPR/Cas9. Even though it is easier to construct DNAs for sgRNA synthesis than it is to make a pair of TALENs, this screening procedure reduces the advantage of CRISPR/Cas9. This low probability of designing a good sgRNA is not always supported by the subsequent research [27]. In that report, a total of 83 sgRNAs targeting 23 genes were examined using the Illumina MiSec platform. The authors showed that 81 sgRNAs introduced mutations into their target loci. The higher yield of sgRNAs capable of mutation introduction compared to that in our study is probably attributable to the differences in the methods used to detect mutations. The Cel-1-mediated method would be less sensitive than the sequencing-based method, and we ignored mutations that occurred at very low rates. Indeed, Gandhi et al. [24] reported that 40% of sgRNAs (33 of 83) had mutation rates under 20%.

The second limitation to the use of CRISPR/Cas9 in *Ciona* is that even though we obtained a guide RNA capable of introducing mutations, the mutation rate is generally lower than those of good TALENs. In our 2014 report, two sgRNAs exhibited mutation efficiencies of 67.7–75.9%. In contrast, a good TALEN pair has an 80–100% mutation efficiency [17]. This score cannot be disregarded, because the probability of biallelic mutations, which is essential for phenotype appearance, depends on its squared score. Similar results were reported by Gandhi et al. [24]. In this report, the mutation rate of sgRNAs ranged from 0 to 59.6%. The two reports suggest that TALENs are superior to CRISPR/Cas9 in terms of the reliability of mutation rates for knockout genes [36–44]. Therefore, we continued to use TALENs even though their construction is more time-consuming than that of CRISPR/Cas9.

Recently, improved mutation rates with CRISPR/Cas9 were achieved in zebrafish [45]. That report showed that (1) an additional nucleotide at the 5′ end of gRNAs, which are supplied to supplement the T7 promoter or added by RNA polIII, strongly reduces mutation rates and that (2) it is better to separate crRNA from tracrRNA, meaning that single-guide RNAs (sgRNAs) have lower mutation efficacies than crRNAs+tracrRNA (*see* **Note 2**).

In the present study, the authors used custom-made RNAs and Cas9 proteins supplied by the Integrated DNA Technologies (IDT) to achieve both improvements. When we used IDT products, mutation efficacies were greatly improved (Fig. 2). We tested 36 crRNAs, of which 19 had mutation rates higher than 80% (Fig. 2). Reeves et al. [31] also used products from IDT and successfully captured functions of the transcription factor gene *brachyury*. Therefore, we strongly recommend this improved method for enhanced mutagenesis in *Ciona*. The detailed experimental procedures are described in the next section.

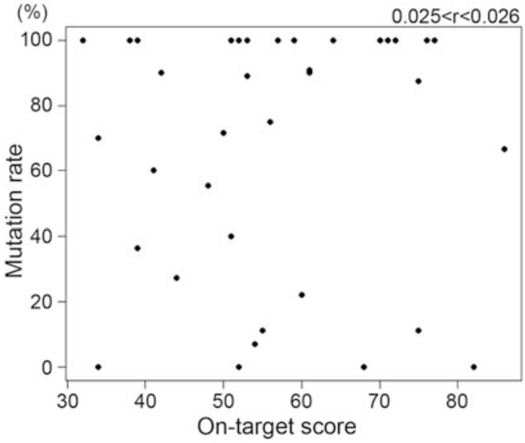

Fig. 2 The relationships between the on-target scores and the mutation rates of 36 crRNAs. The mutation rates were estimated by sequencing the cloned PCR fragments of the target sites. The "*r*" exhibits the Pearson correlation coefficient

The new method of CRISPR/Cas9-based mutagenesis requires microinjection [46], which is a more difficult and time-consuming technique than electroporation. However, microinjection assures the introduction of nucleic acids in a less mosaic fashion than electroporation. With electroporation, more than a few embryos lack transgenes, and for those that do have them, their quantities among blastomeres are highly mosaic. Therefore, we need to select animals whose transgenes have less mosaicism by referring to the expression level of a reporter gene prior to observing phenotypes. This step is unnecessary in the microinjection-based method, because we can check the successful introduction of solutions into eggs under microscope. Therefore, we think that the time efficiency issue is not necessarily a disadvantage of microinjection.

2 Materials

2.1 Animals

Wild-type *Ciona intestinalis* type A can be collected from fishery harbors. In Japan, the National BioResource Project (NBRP) provides cultivated closed colonies of this species. The genome sequence of this population is also available from the genome database (http://ghost.zool.kyoto-u.ac.jp/). The *Ciona* genome has more than 1% nucleotide variations between haplotypes [5]. The variations negatively influence the mutation rates when they are present at the target site of a guide RNA. When designing a guide RNA, we can reduce this risk by checking the DNA sequences in the population against the NBRP wild type.

2.2 Guide RNAs and Cas9

We use custom-made Alt-R® CRISPR-Cas9 crRNA. This version has modifications at the ends to resist degradation by RNase. Our designed crRNAs always recognize exactly 20 nucleotides of target sites. The IDT homepage has a program to custom-design crRNAs. This program provides an "on-target score," which is an estimation of mutation efficacies. However, our results suggest that the score does not always guarantee good mutation rates in *Ciona* (Fig. 2). Because crRNAs require tracrRNA to make a complex with Cas9, IDT provides Alt-R® CRISPR-Cas9 tracrRNA.

IDT also provides Cas9 protein. We use Alt-R® S.p. Cas9 Nuclease V3. In vitro synthesized *Cas9* mRNA can also be used. However, a previous report in zebrafish suggests that RNP formation prior to microinjection significantly increases mutation efficiency compared to supplying *Cas9* as mRNA [47].

2.3 Reagents

1. Dechorionation solution: 1% sodium thioglycolate and 0.05–0.1% actinase E (a kind of protease) in seawater. Store at 4 °C. Long-term storage is not recommended.

2. 2 M NaOH.
3. Genomic DNA extraction kit such as Wizard Genomic DNA Purification Kit (Promega).
4. 15% polyacrylamide gel in 1× TAE.
5. In-Fusion cloning kit.
6. RNase-free H_2O.
7. 100% isopropanol.
8. RNase-free Duplex buffer.
9. RNase-free 10 mg/mL Fast Green FCF.
10. RNase-free 10 mg/mL glycogen.
11. 0.77 M mannitol.
12. Seawater.

2.4 Apparatus

1. Three-axis coarse manipulator.
2. Objective microscope.
3. Electroporator and 4 mm width cuvettes.

2.5 Database and Website

The genome and genetic information of *Ciona* is available in the Ghost (http://ghost.zool.kyoto-u.ac.jp/default_ht.html) and Aniseed (http://www.aniseed.cnrs.fr/) databases. To help researchers use genome editing techniques, we developed a website (http://marinebio.nbrp.jp/ciona/forwardToKnockOutAction.do) to provide information related to genome editing in *Ciona*. The website deals with our vector sequences of TALEN and CRISPR/Cas9, the genes that are so far targeted by genome editing, target sites of the genes, primers for detecting mutations, gel images detecting mutations, sequences of mutated genes, and references. Expression vectors can be requested from the website.

3 Methods

3.1 Dechorionation of Eggs

1. Unfertilized eggs collected from the wild type are dechorionated with dechorionation solution. Add 200 μL of 2 M NaOH to 10 mL dechorionation solution before use. Gently pipette to dissolve the white cloudy precipitate that forms. Enter eggs into the prepared dechorionation solution, and mix them by occasional pipetting. Removal of chorion should be confirmed with a microscope.
2. After dechorionation (*see* **Note 3**), wash naked eggs several times by exchanging seawater (*see* **Note 4**), followed by insemination. The occurrence of fertilization can be seen by the shape change of eggs.

3.2 Microinjection of CRISPR/Cas9 Components

1. Prepare for microinjection solution as follows:

RNase-free H_2O	9.2 μL
Duplex buffer	2 μL
crRNA (250 ng/μL)	2 μL
tracrRNA (1 μg/μL)	2 μL
Total	15 μL

Heat at 95 °C for 5 min.

Cool to room temperature (RT).

Add 0.8 μL of Cas9 protein solution (10 μg/μL).

Incubate for 5 min at RT.

Add 4 μL of Fast Green FCF (RNase-free treated, 10 μg/μL).

Divide this solution into 2 μL aliquots and store at −80 °C until use.

2. The detailed method of microinjection into *Ciona* eggs is described in [46]. We microinject solutions into unfertilized eggs because unfertilized eggs can be kept stationary for a long time compared to fertilized eggs, which exhibit their first cleavage in 1 h.
3. After microinjection, inseminate eggs to fertilize them. Observe shape changes by the first and second rounds of ooplasmic segregations [48], which are the signature of fertilization. Incubate embryos at 18 °C.

3.3 Examining Mutation Efficiency

Before starting thorough analyses, we strongly recommend measuring the mutation rate. This is because we can estimate the probability of the phenotype appearance based on this score:

1. Pick up about 20–100 injected larvae. Collect them in a 1.5 mL tube. Centrifuge briefly and discard excess seawater.
2. Extract genomic DNA from the gathered larvae in bulk. We use the Wizard genomic DNA purification kit to isolate genomic DNA. Dissolve collected larvae in 600 μL of nuclei lysis solution. Incubate for several hours at 50 °C. Then precipitate proteins with 200 μL protein precipitation solution according to the manufacturer's instructions (both solutions are included in the Wizard kit). Add glycogen or an equivalent reagent to enhance the precipitation with isopropanol. After the isopropanol precipitation, dissolve the DNA in 10–20 μL water.
3. A 200–300 base pair amplicon containing the target site of a guide RNA is amplified by PCR. The PCR fragment is analyzed by means of polyacrylamide gel electrophoresis (PAGE) with 15% gel [49] (*see* **Note 5**). The PAGE-based method is highly recommended because we can apply PCR products directly

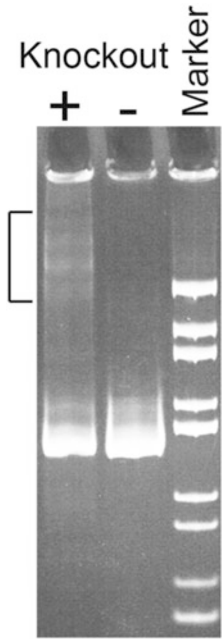

Fig. 3 Gel electrophoreses to monitor mutations induced by CRISPR/Cas9. The results of the polyacrylamide gel-mediated method. + Lanes exhibit PCR products derived from animals in which the target gene is knocked out with CRISPR/Cas9, and lanes exhibit PCR products from control animals. The bands suggesting mutations are indicated by the bracket

without any posttreatment, and the results are easier to interpret. When a considerable number of bands appear at the high molecular weight region (Fig. 3), the remaining PCR products are purified by agarose gel electrophoresis, cloning, and sequencing after subcloning into a conventional vector. This process is necessary to calculate mutation rates.

3.4 Tissue-Specific Knockouts

In *Ciona*, genome editing methods are mainly used to analyze gene function at the G0 generation. Microinjection of guide RNAs and Cas9 protein results in the ubiquitous knockout of target genes, ideally in all blastomeres. Many genes have multiple expression domains. When a phenotype appears after genome editing, the expression domain responsible for the phenotype must be identified for a better understanding of the underlying mechanisms. For this purpose, tissue-restricted knockout of a gene is an effective strategy. This is particularly important when a gene of interest is expressed at two different developmental stages. If the gene has a crucial role at the earlier stage, we cannot address the function at the later stage due to death or arrest of development.

Conditional tissue-specific knockout is a reliable way to overcome this limitation. An advantage of *Ciona* is the availability of various tissue-specific expression constructs (Fig. 4) [9, 11]. By

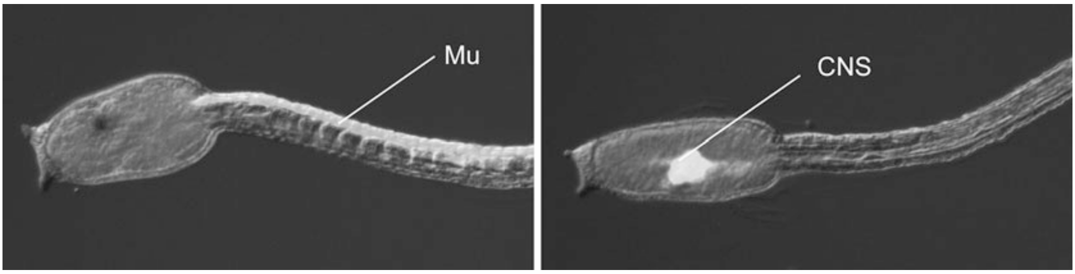

Fig. 4 Tissue-specific expression of fluorescent protein in *Ciona* larvae. The left larva expresses mCherry in the muscle (Mu), and the right larva expresses mCherry in the central nervous system (CNS)

expressing sgRNAs and Cas9 from expression vectors, tissue-limited functional analyses of genes have been reported [22]. With the new CRISPR/Cas9 method, in contrast, it is more difficult to carry out tissue-specific knockouts of genes, especially at the later developmental stage. This is because we cannot use expression vectors for guide RNAs, as polIII promoters add additional nucleotides at the 5′ end of the transcripts [50], which will reduce mutation rates. Although we have not tested this, Cas9 could be supplied from an expression vector in the new method to achieve tissue-specific knockouts. At the embryonic stages, sufficient quantities of microinjected crRNAs and tracrRNAs remain for knockout at any tissue. At the later developmental stage, however, tissue-specific knockout will be unfeasible since microinjected crRNAs and tracrRNAs are exhausted due to dilution by increasing cell number, growth, and degradation.

For efficient tissue-specific knockout of genes, TALENs have more advantages than CRISPR/Cas9. We developed TALEN expression vectors for the epidermis, nervous system, muscle, endoderm, and mesenchyme [41]. Using these vectors, we identified the responsible tissues in which genes of interest function. Expression vectors of TALENs can be introduced by both microinjection and electroporation, which is another advantage over CRISPR/Cas9 for conditional knockout. The constructed TALEN assemblies can also be used for ubiquitous knockout by subcloning into the vector for in vitro mRNA synthesis (Fig. 5). If your laboratory environment allows, we still favor TALENs to observe phenotypes in detail.

3.5 Construction of TALENs

We recommend using Platinum TALENs for knockouts in *Ciona* [20], because TALENs generated with this kit have a high probability of producing a good mutation rate. Our rough estimation suggests that about 70% of constructed TALENs have mutation rates sufficient for functional analyses of genes in the G0 generation and germline mutagenesis. The TALEN repeat arrays can be assembled in vectors compatible for expression systems in *Ciona* (Fig. 4) [17]. Vector information is available at our website which is http://marinebio.nbrp.jp/ciona/forwardToKnockOutAction.do.

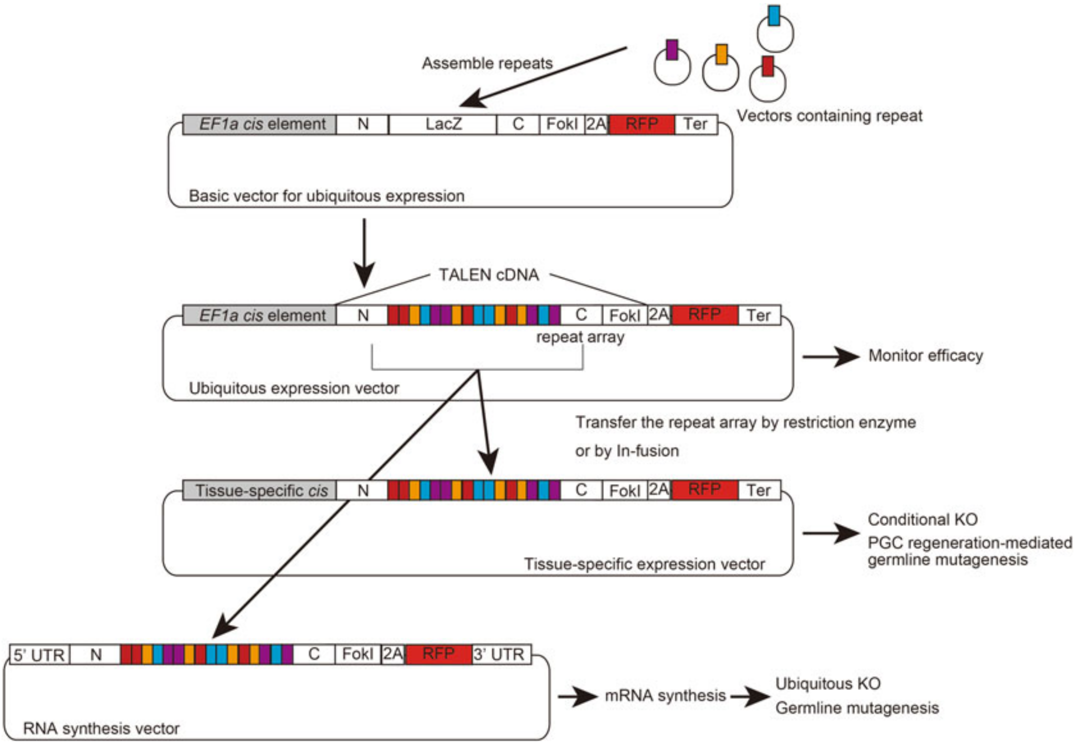
Assemble repeats
Vectors containing repeat
EF1a cis element
N
LacZ
C
FokI
2A
RFP
Ter
Basic vector for ubiquitous expression
TALEN cDNA
repeat array
Ubiquitous expression vector
Monitor efficacy
Transfer the repeat array by restriction enzyme or by In-fusion
Tissue-specific cis
Tissue-specific expression vector
Conditional KO
PGC regeneration-mediated germline mutagenesis
5' UTR
3' UTR
RNA synthesis vector
mRNA synthesis
Ubiquitous KO
Germline mutagenesis

Fig. 5 Our TALEN vector systems for gene knockouts in *Ciona*. Ter, transcription termination sequence; RFP, cDNA encoding red fluorescent protein; and LacZ, a portion of *LacZ* cDNA. N, cDNA encoding amino terminal region of TALEN; C, cDNA encoding carboxyl terminal region of TALEN; and FokI, cDNA encoding the nuclease domain of *FokI*. 2A, 2A peptide for polycistronic polypeptide synthesis. 5′ UTR and 3′ UTR, untranslated regions from cDNA encoding beta tubulin of *Halocynthia roretzi* [51]

3.6 Electroporation of Vectors

Constructed TALENs can be introduced into *Ciona* embryos by electroporation [9, 52]. An example of the several methods of electroporation follows:

1. Dechorionate unfertilized eggs as described in Subheading 3.1.
2. After fertilization, wash the eggs once with a 1:9 dilution of seawater and 0.77 M mannitol (mannitol-seawater solution).
3. Mix 30 μg of each plasmid DNA harboring paired left and right TALEN cDNAs in 500 μg of mannitol-seawater solution in a cuvette (4 mm width) with a glass pipette. Then enter 300 μg of the mannitol-seawater solution containing fertilized eggs (prepared in **step 3**) into the cuvette. Mix by gentle pipetting. Usually we do not adjust the number of eggs for electroporation.
4. The cuvette is set in the machine (we use GenePulser II), and then a pulse is given under the conditions of 50–52 V for 20 ms. Usually we provide a pulse 20–25 min after fertilization at 18 °C.
5. Eggs are washed several times with seawater and cultured at 16–18 °C.

3.7 Germline Mutagenesis

Both TALENs and CRISPR/Cas9 can introduce mutations in germ cell genomes as well as in somatic cell genomes. We can establish mutant lines by culturing animals developed from the mutated germ cells. In our previous report [53], TALEN mRNAs are introduced into embryos by means of microinjection to establish mutants. The same strategy can be applied to CRISPR/Cas9.

Although simple, this method has two limitations. One is that the mutation rate in the germline genome is significantly lower than those in somatic cell genomes. This is probably due to the small size of primordial germ cells (PGCs) during embryogenesis. After a few rounds of unequal cleavages, a pair of posteriormost vegetal cells is destined to be PGCs [54]. These paired cells are very small, indicating that injected mRNAs are not distributed efficiently in the PGCs, which reduces the chance of gene mutation. The other limitation is the unavailability of germline-specific knockouts, because the *cis* element inducing gene expression in germ cells has not been isolated in *Ciona*. When a target gene has an essential role during development, its knockout by microinjection results in the emergence of phenotypes, and further growth is inhibited, resulting in the death of animals before reproductive maturation. By using methods to induce gene knockout in a ubiquitous fashion, we can generate only mutant lines of genes that do not cause any serious effect during development.

Recently, we reported a method that overcomes these limitations by combining tissue-specific knockout by TALENs and germline regeneration [55]. At the larval stage, *Ciona* PGCs are present in the posterior part of the tail and can be removed easily by amputation with a scalpel. When tail-amputated larvae develop into adults, the adults have germ cells that are regenerated from somatic cells [56, 57]. Because somatic cells have much higher mutation rates than original PGCs, the regenerated germ cells also have high mutation rates. Therefore, this germline regeneration method improves screening efficiency to establish mutant lines.

Germline regeneration enables us to establish mutants through the electroporation of expression vectors of TALENs [55, 58]. When TALENs are expressed in the epidermis, the nervous system, or muscle and then the PGCs of these animals are removed by tail amputation, the regenerated PGCs have a chance to possess mutations that the next generation can inherit. By expressing TALENs in a tissue whose target gene has no major function, we can establish mutant lines of genes even though the genes have essential roles during development. Indeed, we reported the creation of mutants of *Hox12* through the germline regeneration method. Because this gene is necessary for the formation of the digestive tube, *Hox12*-deficient animals die within 4 weeks if the conventional method was operated.

4 Notes

1. A paper [59] asserts that Tunicata, Vertebrata, and Cephalochordata can be regarded as a phylum in the branch of deuterostomes.
2. We have controversial data that sgRNAs have higher mutation rates than crRNAs+tracrRNAs targeting the same sites.
3. Usually it takes a few minutes for dechorionation at 18 °C. The time varies by subtle changes in the room temperature and seasonal differences in *Ciona* eggs.
4. There are two ways to perform this step. One is to gently swirl a petri dish so that dechorionated eggs accumulate in the center. Using a glass pipette, collect the eggs and transfer them to another petri dish filled with seawater. The other way is to use a manual centrifuge. Centrifuging too vigorously decreases the quality of the eggs.
5. Our electrophoresis conditions are 100 V for 2–2.5 h at room temperature. Higher voltages may disturb DNA bands.

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Chapter 29

Genome Editing of *C. elegans*

Takuma Sugi

Abstract

Caenorhabditis elegans, a 1 mm long free-living nematode, is a traditional model animal for genetic investigations of various biological processes. Characteristic features that make *C. elegans* a powerful model of choice for eukaryotic genetic studies include its rapid life cycle, well-annotated genome, simple morphology, and transparency. Recently, genome editing technologies have been increasingly used in *C. elegans*, thereby facilitating their genetic analyses. Here, I introduce a protocol frequently used in *C. elegans* genome editing.

Key words *C. elegans*, CRISPR/Cas9, Homology-dependent repair

1 Introduction

The nematode *Caenorhabditis elegans* became a powerful model organism just over 50 years ago [1]. This tiny animal has greatly contributed to investigations of the functions of genes in development and cellular biology. One advantage of using *C. elegans* as a model animal is its simple morphology; the adult hermaphrodite is composed of only 959 somatic cells, including 302 neurons, and cell number does not vary among individuals. Researchers have tracked the fate of every cell, from fertilization to adulthood, in living animals and have generated a complete cell lineage [2]. Features that make the nematode highly amenable to laboratory research also include its small size, short life cycle, and ability to survive long-term freezing for storage. These advantageous features have allowed *C. elegans* researchers to uncover novel biological mechanisms, such as apoptosis, and develop novel techniques, such as green fluorescent protein (GFP) tagging, in the life sciences research field.

C. elegans is a genetically tractable model system, for which many genetic resources and tools have been developed. There are approximately 20,359 protein-coding genes in *C. elegans*

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(WormBase referential freeze WS250, November 2015). In OrthoList, a compendium of *C. elegans* genes with human orthologs, 7663 protein-coding genes are predicted to have human orthologs, corresponding to approximately 38% of the *C. elegans* genome [3].

Microinjection of a gene of interest has been an indispensable genetic technique to study gene functions in *C. elegans*. The microinjection of genes including fluorescent protein-coding genes into the gonad of the adult hermaphrodite results in the formation of semi-stable extrachromosomal arrays that are comprised of many copies of the injected DNA [4]. The transgenes, which are typically overexpressed in somatic tissues and silenced in the germline and early embryo, can be integrated into the genome by UV irradiation or microparticle bombardment [5, 6]. These integration methods have allowed the generation of low-copy transgenes, which are expressed at closer to endogenous levels [7]. However, this experiment is less efficient, time-consuming, and not easily able to control genomic location for gene insertion. Thus, a strategy for inducing a stable gene knock-in at a desired genomic location has been required in the nematode research field.

Due to these requirements, *C. elegans* researchers have recently utilized targeted genome editing technologies. A large number of protocols for genome editing technologies have been established since Wood et al. reported a landmark paper on genome editing in *C. elegans* [8–10]. These protocols include several strategies for homology-dependent repair (HDR)-mediated gene knock-in of *C. elegans* genome. In particular, Dickinson et al. have established a versatile and simple strategy for precise gene insertion without an exogenous sequence at the targeted locus or marker mutations [11, 12]. They have designed a plasmid that includes a fluorescent tag gene and the self-excising cassette (SEC) carrying a drug resistance gene, a visible phenotypic marker, and a heat-shock-inducible Cre recombinase gene. Incorporation of the SEC from the plasmid into genomic loci permits easy excision of unwanted sequences upon heat-shock treatment after identification of the desired mutant using drug selection and screening for a visible phenotype.

Here, I introduce this protocol by applying it to insert the fluorescent protein TagRFP-T into the genomic region downstream of the promoter of *glr-1* gene [13, 14] (a *C. elegans* AMPA-type receptor ortholog) (Fig. 1).

2 Materials

2.1 Plasmids

1. The CRISPR guide RNA selection tool website (http://genome.sfu.ca/crispr/about.html).
2. Cas9 and sgRNA co-expression plasmid: Purchase the pDD162 vector (Addgene, #47549) or pJW1219 vector

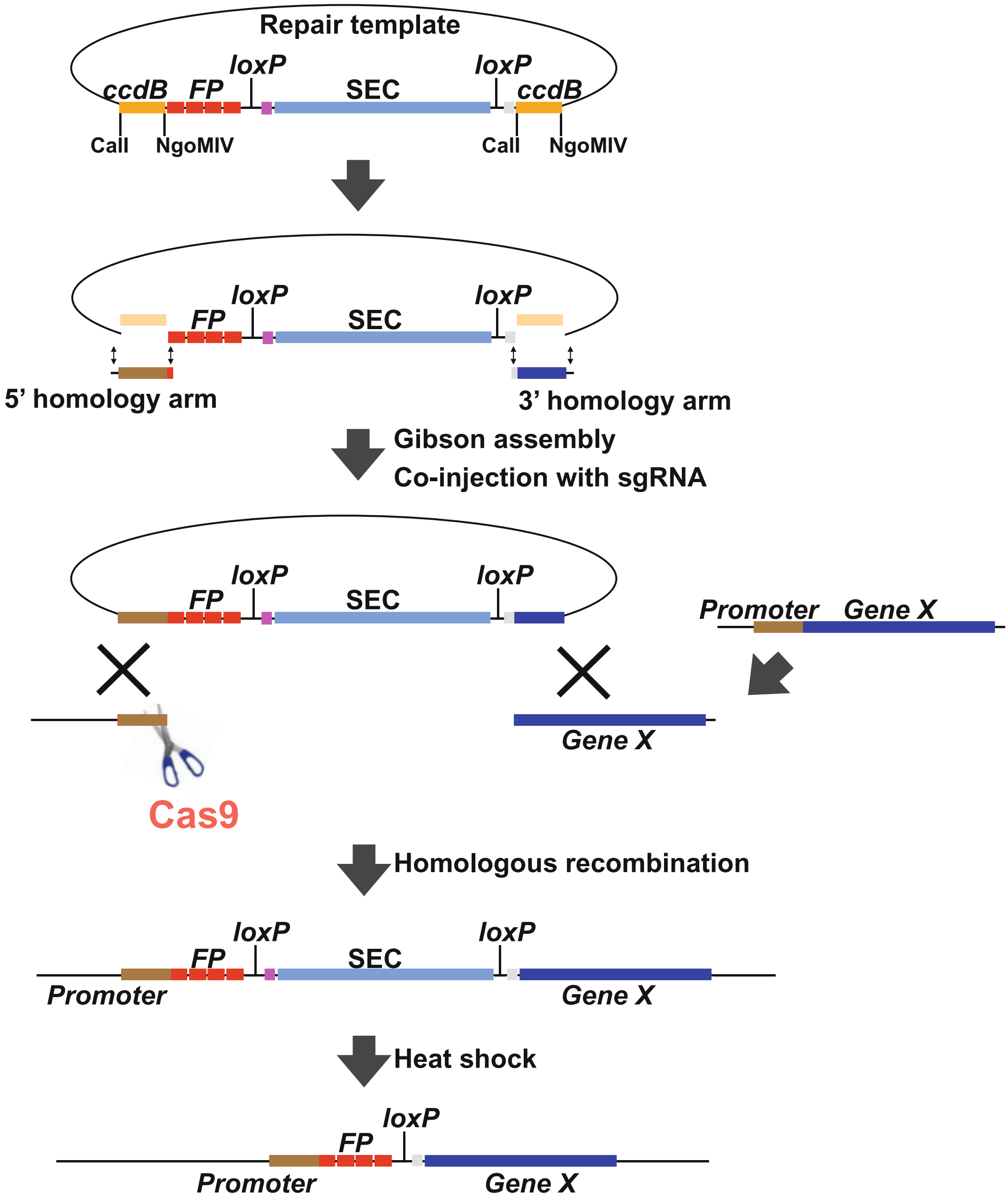

Fig. 1 Schematic of homologous recombination-mediated *FP* insertion using CRISPR/Cas9 system. The self-excising cassette is composed of a drug resistance gene, a visible phenotypic marker, and a heat-shock-inducible Cre recombinase gene. The SEC selection marker in the resulting *FP*-inserted worms can be excised by heat-shock treatment of worms

(Addgene, #61250) to co-express Cas9 and sgRNA under the control of R07E5.16 *U6* promoter. pJW1219 vector is a pDD162 derivative with the higher efficiency.

3. Q5 Site-Directed Mutagenesis Kit.
4. Forward primer 1: 5′- N_{20} GTTTTAGAGCTAGAAATAGCAAGT-3′.

   Reverse primer 1: 5′-CAAGACATCTCGCAATAGG-3′.

   Forward primer 2: 5′-ATCGACCGTAAGCGTTACACCTCCACCAAGG-3′.

   Sequencing primer 1: 5′-GGTGTGAAATACCGCACAGA-3′.

   M13 forward primer: 5′-TGTAAAACGACGGCCAGT-3′.

   M13 reverse primer: 5′-CAGGAAACAGCTATGACCATG-3′.
5. Homologous repair template plasmid: Several homologous repair templates are available for knock-in of a gene encoding fluorescent protein. pDD286 plasmid for knock-in of TagRFP-T was chosen here and purchased from Addgene as transformed bacteria. Streak bacteria onto an ampicillin plate. Pick a single colony and culture it at 30 °C for 16 h. Purify the plasmid DNA using miniprep kit.
6. Restriction enzymes: ClaI and NgoMIV.
7. Purification of PCR products: QIAquick PCR Purification Kit.
8. Gibson assembly: NEBuilder HiFi DNA Assembly Cloning Kit.
9. Injection marker: Several plasmids for germ transformation in *C. elegans* are available. pCFJ104 (Addgene, #19328) for expressing mCherry in body wall muscles under the control of the *myo-3* promoter were used here.

2.2 C. elegans Preparation

1. *C. elegans*: Obtain the wild-type N2 strain from *Caenorhabditis Genetics Center*. Maintain and handle the wild-type N2 strain using standard methods [1].
2. Nematode Growth Medium (NGM) plate: Weigh 6 g NaCl, 40 g agar, and 5 g peptone and transfer to the 3 L flask. Add 1950 mL of water and autoclave at 120 °C for 20 min. Cool to approximately 60 °C and add 2 mL of 1 M $CaCl_2$, 2 mL of 1 M $MgSO_4$, and 50 mL of 1 M KPO_4 solution (108.3 g KH_2PO_4 and K_2HPO_4 in water). Pour into a 60 mm Petri plate. Streak *Escherichia coli* OP50 propagated at 37 °C for 12 h.

3 Methods

3.1 DNA Construction for Cas9-sgRNA Plasmid

1. Choose the target site and submit the genomic sequence to the CRISPR guide RNA selection tool website.
2. The tool returns target sites of the form 5′ N_{20}-NGG-3′, where N is any base. The GG at the 3′ end of each guide are known to

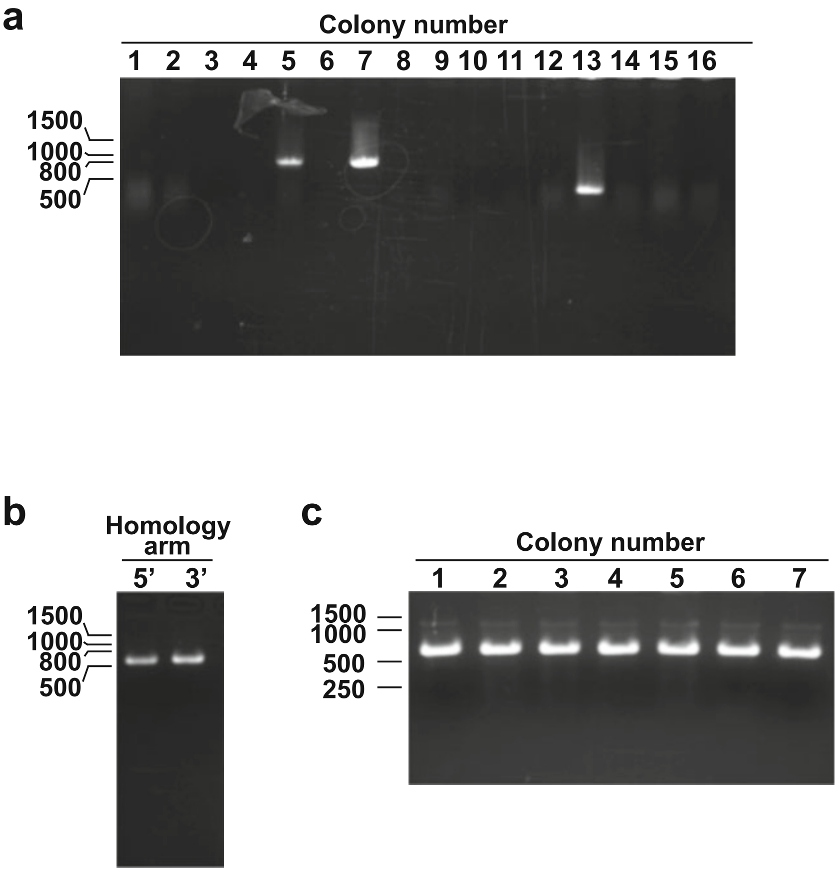

Fig. 2 Electrophoresis check of the DNA construction process. (**a**) Identification of an *E. coli* colony carrying the appropriate Cas9-sgRNA plasmid by colony PCR and agarose electrophoresis analysis. This analysis was performed using forward primer that anneals to the sequence of the original pDD286 vector and reverse primer that anneals to the inside of the forward primer used in the original PCR. (**b**) Agarose electrophoresis of PCR products for amplification of the 5′ and 3′ homology arms. (**c**) Identification of a colony carrying the appropriately assembled plasmid by colony PCR. The M13 forward primer and the second primer used for amplifying a homology arm were used for the analysis and agarose electrophoresis analysis

have higher efficiency for NGG PAM [15]. Insert the N_{20} sequence into the pDD162 or pJW1219. I used pDD162 here.

3. Transform the fragment created by PCR using the forward primer 1, reverse primer 2 (*see* **Note 1**) and Q5 Site-Directed Mutagenesis Kit to suitable competent cells, and culture it on an ampicillin plate for 14 h.
4. Identify the colony carrying the appropriate Cas9-sgRNA plasmid by colony PCR using forward primer 2 that anneals to the sequence of the original pDD286 vector and a reverse primer 2 that anneals to the inside of the forward primer used in the above PCR (Fig. 2a).
5. Sequence the resultant plasmid using sequencing primer 1.

3.2 DNA Construction for Homologous Recombination Template

1. Digest the FP-SEC vector using one of two pairs of restriction enzymes recommended by Dickinson et al. [12] (*see* **Note 2**). ClaI and NgoMIV were used here, because the 5′ homology arm could be fused directly to the fluorescent protein TagRFP-T, without additional sequence.
2. Choose the genomic regions suitable for homology arms (500–700 bp long), and design the primers to assemble homology arms into the FP-SEC vector (pDD286; Fig. 1). The homology arms are inserted in place of the *ccdB*-negative selection markers, which are flanked by restriction sites. Because *E. coli* DH5α harboring the original plasmid expression cannot grow on the usual ampicillin plate due to the *ccdB* marker, successful insertion should be verified by picking a single colony that grows on the plate.
3. Using the two sets of the primers, generate two PCR products by amplifying homology arms with sequences overlapping with sequences of the FP-SEC vector (Fig. 2b) (*see* **Note 3**).
4. Mix the PCR products and purify them using QIAquick PCR Purification Kit. Elute the purified PCR products with 35 μL of autoclaved water.
5. Assemble the purified PCR products with FP-SEC vector using NEBuilder HiFi DNA Assembly Cloning Kit. Mix 2 μL of PCR products, 0.5 μL of vector, and 2.5 μL of NEBuilder HiFi DNA Assembly Master Mix. Incubate this mixture at 50 °C for 1 h. Transform 2 μL of the reaction to NEB 5-α-competent cells.
6. Perform colony PCR using the M13 forward primer and the second primer used for amplifying a homology arm to identify a colony expressing appropriately assembled plasmid (Fig. 2c).
7. Sequence the resultant plasmid using the M13 forward and reverse primers.

3.3 Injection

1. Prepare the injection solution:

   10 ng/μL homologous repair template

   50 ng/μL Cas9-sgRNA plasmid

   Suitable co-injection marker (5 ng/μL pCFJ104 for expressing mCherry in body wall muscles under the control of *myo-3* promoter was used here) (*see* **Note 4**).
2. Inject the solution into the gonads of a few young adult worms using standard methods [4].
3. Cultivate the injected worms on the NGM plate at 25 °C for 2.5 days.

3.4 Screening

1. Dissolve hygromycin in water to prepare a 5 mg/mL hygromycin solution. Pour 500 μL of this solution onto the surface of each NGM plate in which worms were cultivated for

2.5 days. Swirl so that the solution covers the entire surface of the plate and let it dry.

2. Identify worms that survive the antibiotic screening and exhibit roller phenotype. Among them, transfer worms that do not carry co-injection marker (e.g., *myo-3p::mCherry*) as an extrachromosomal array individually to new NGM plates. The NGM plate does not need to contain antibiotic such as hygromycin. Cultivate worms at 20 °C for a few days.
3. Find an NGM plate on which all adult worms have roller phenotype. These worms should be homozygous knock-in animals. Transfer 5–10 L1/L2 worms from this plate to another new NGM plate.
4. To remove SEC, induce expression of *heat-shock promoter::cre* by heat-shock treatment of worms. Place the NGM plate at 32 ° C for 20 min and allow them to recover at 20 °C.
5. Pick a few knock-in worms that do not exhibit roller phenotype and maintain them for each experiment.

4 Notes

1. The primers used for creating the Cas9-sgRNA plasmid should not include the PAM sequence.
2. The pair of restriction enzymes used for FP-SEC vector construction should be chosen according to each FP-SEC vector and application. For example, if AvrII and NgoMIV are used with pDD286, the repair template includes a flexible linker between the 5′ homology arm and TagRFP-T. This combination is suitable for generating C-terminal tags. On the other hand, if ClaI and NgoMIV are used, the 5′ homology arm will be fused with directly to the TagRFP-T, without additional sequence. This is useful for creating N-terminal tags without flexible linker.
3. Design a primer for creating FP-SEC vector so that FP-SEC insertion disrupt the sgRNA target site. Ideally, the insertion site is within 10 bp of the PAM sequence. Otherwise, use a primer that introduce silent mutations to prevent Cas9 from cutting the repair template.
4. 50 ng/μL pKDK66 can be used as another injection marker for expressing EGFP in the intestine under the control of *ges-1* promoter.

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